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(54) Title: REAGENTS AND METHODS FOR DIAGNOSIS AND PROGNOSIS OF PROLIFERATIVE DISORDERS			
(57) Abstract <p>The present invention relates to diagnostic and prognostic assays which assess the phenotype and aggressiveness of a disorder by detecting the stability of a cyclin kinase inhibitor (CKI) protein.</p>			

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Reagents and Methods for Diagnosis and Prognosis of Proliferative Disorders

Background of the Invention

The cell division cycle is one of the most fundamental processes in biology which, in multicellular organisms, ensures the controlled generation of cells with specialized functions. Under normal growth conditions, cell proliferation is tightly regulated in response to diverse intra- and extracellular signals. This is achieved by a complex network of protooncogenes and tumor-suppressor genes that are components of various signal transduction pathways. Activation of a protooncogene(s) and/or a loss of a tumor suppressor gene(s) can lead to the unregulated activity of the cell cycle machinery. This, in turn, will lead to unregulated cell proliferation and to the accumulation of genetic errors which ultimately will result in the development of cancer (Pardee, *Science* 246:603-608, 1989).

In the eukaryotic cell cycle a key role is played by the cyclin-dependent kinases (CDKs). CDK complexes are formed via the association of a regulatory cyclin subunit and a catalytic kinase subunit. In mammalian cells, the combination of the kinase subunits (such as cdc2, CDK2, CDK4 or CDK6) with a variety of cyclin subunits (such as cyclin A, B1, B2, D1, D2, D3 or E) results in the assembly of functionally distinct kinase complexes. The coordinated activation of these complexes drives the cells through the cell cycle and ensures the fidelity of the process (Draetta, *Trends Biochem. Sci.* 15:378-382, 1990; Sherr, *Cell* 73:1059-1065, 1993). Each step in the cell cycle is regulated by a distinct and specific cyclin-dependent kinase. For example, complexes of CDK4 and D-type cyclins govern the early G1 phase of the cell cycle, while the activity of the CDK2/cyclin E complex is rate limiting for the G1 to S-phase transition. The CDK2/cyclin A kinase is required for the progression through S-phase and the cdc2/cyclin B complex controls the entry into M-phase (Sherr, *Cell* 73:1059-1065, 1993).

The CDK complex activity is regulated by mechanisms such as stimulatory or inhibitory phosphorylations as well as the synthesis and degradation of the kinase and cyclin subunits themselves. Recently, a link has been established between the regulation of the activity of cyclin-dependent kinases and cancer by the discovery of a group of CDK inhibitors including the p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, p19/p20^{INK4d}, p21^{Waf1/Cip1}, p27^{Kip1} and p57^{Kip2} proteins. The activity of p21 is regulated transcriptionally by DNA damage through the induction of p53, senescence and quiescence (Harper et al., *Cell* 75:805-816, 1993). The inhibitory activity of p27 is induced by the negative growth factor TGF- β and by contact inhibition (Polyak et al., *Cell* 78:66-69, 1994). These proteins, when bound to CDK complexes, inhibit their kinase activity, thereby inhibiting progression through the cell cycle. It is generally accepted that binding of these inhibitors to the CDK/cyclin complex prevents its

activation. Alternatively, these inhibitors may interfere with the interaction of the enzyme with its substrates or its cofactors.

While p21 and p27 inhibit all the CDK/cyclin complexes tested, the *INK4* proteins, e.g., p16, p15, p18 and p19 block exclusively the activity of the CDK4/cyclin D and CDK6/cyclin D complexes in the early G1 phase (Serrano et al., *Nature* 366:704-707, 1993), by either preventing the interaction of CDK4 and Cyclin D1, or indirectly preventing catalysis. As mentioned above, the p21 is positively regulated by the tumor suppresser p53 which is mutated in approx. 50% of all human cancers. p21 may mediate the tumor suppresser activity of p53 at the level of cyclin-dependent kinase activity. p16 is the product of a tumor suppresser gene localized to the 9p21 locus, which is frequently mutated in human cancer cells.

Summary of the Invention

One aspect of the present invention relates to diagnostic assays for determining, in the context of cells isolated from a patient, the protein level of a CDK inhibitor, which level can be a useful diagnostic/prognostic marker for risk assessment and phenotyping cell and tissue samples. As described herein, the subject assay provides a method for determining if an animal is at risk for a disorder characterized by aberrant cell proliferation, differentiation and/or apoptosis, and also may be used for prognostic purposes when such aberrant cell phenotypes are known.

The subject method can be used for diagnosing a hyperproliferative disorder in a patient which disorder is associated with the destabilization of a CKI protein, such as p27^{kip1}, in cells of the patient, comprising: (i) ascertaining the level of a CKI protein in a sample of cells from the patient; and (ii) diagnosing the presence or absence of a hyperproliferative disorder utilizing, at least in part, the ascertained level of the CKI protein, wherein a reduced level of CKI protein in the sample, relative to a normal control sample of cells, correlates with the presence of a hyperproliferative disorder. In another embodiment, the subject method is a prognostic method for evaluating a cancer patient's risk of death and/or recurrence of a cancer, comprising (i) ascertaining the level of a CKI protein in a sample of cancer cells from the patient; and (ii) predicting the patient's risk of death and/or recurrence of a cancer utilizing, at least in part, the ascertained level of CKI protein, wherein a reduced level of CKI protein in the sample, relative to a normal control sample of cells, correlates with an increased risk of death and/or recurrence of a cancer.

The present invention also provides a test kit for detecting stability CKI proteins comprising an antibody, or fragment thereof, which selectively binds to a CKI protein, e.g., the antibody selectively binds to the p27 protein of SEQ ID No. 2. In preferred embodiments, the antibody is labeled with a detectable label, e.g., with a label selected from the group consisting

of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. In certain embodiments, the test kit will also include a nucleic acid probe for detecting the amount of CKI transcript in a cell sample.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No. 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Description of the Drawings

Figure 1, panels A-F, illustrates the expression of p27 in normal colon mucosa. Colon mucosae (x200) oriented with the luminal surface toward the top was stained with hematoxylin (a), anti-p27 monoclonal antibody (B), anti-p27 monoclonal antibody blocked by preincubation with purified recombinant p27 (C), anti Ki-67 antibody (E), or probed with p27 cDNA (D). Cells expressing p27 are stained, the nonepithelial cells that appear stained represent intramucosal T lymphocytes (B). Panel F shows an immunoblot of normal mucosa (lanes 1 and 3) and recombinant purified his-tagged p27 (lanes 2 and 4) with anti-p27 monoclonal antibody (lanes 1 and 2) and anti-p27 monoclonal antibody preadsorbed with purified recombinant p27 (lanes 3 and 4).

Figure 2, panels A-F, illustrates p27 expression in tumors in different stages of differentiation. Immunohistochemistry with anti-p27 antibody (x200). (A) well differentiated colon carcinoma, (B) poorly differentiated colon carcinoma, (C) colon carcinoma with well differentiated (right) and poorly differentiated (left) areas. (D-F) moderately differentiated colon carcinoma stained with hematoxylin (D), anti-p27 monoclonal antibody (E), anti-p27 monoclonal antibody blocked by preincubation with purified recombinant p27 (F).

Figures 3A-C illustrate that the lack of p27 is associated with poor survival in patients with colorectal carcinoma. (A) Life table analysis with expression of p27 as absent, weak (1-50% of positive tumors cells), or strong (>50% of positive tumor cells) in total number of tumors; (B) life table analysis with expression of p27 scored as absent or present in total number of tumors; (C) life table analysis with expression of p27 as absent, weak (1-50% of positive tumors cells), or strong (>50% of positive tumor cells) in stage II tumors.

Figure 4 illustrates that p27 mRNA and protein expression do not always correlate. (A-C) p27 mRNA detection by in situ hybridization (400X); (B-D) immunohistochemistry with anti-p27 Mab (400X).

Figure 5 illustrates the kinetics of p27 degradation in colorectal carcinoma samples. Purified recombinant p27 was incubated for the indicated times either alone (A) or in the presence of extracts from tumors expressing high (B), moderate (C) or low (D) p27 levels. Samples in panels E and F were incubates with either proteasome-depleted (E) or proteasome reconstituted (F) extract from a tumor having low p27 protein levels. The reaction products were analyzed by immunoblotting as Pagano et al. (1995) *Science* 269:682-685 with anti-p7 Mab.

Figure 6 is a table showing the correlation between p27 abundance and p27 degradation activity in human colorectal carcinomas. Bank # is the number assigned to the samples at the Deaconess Hospital tumor bank. Protein levels were assessed by immunoblot: +++, detection of a very strong signal; ++, detection of a strong signal; + detection of a very weak signal; -, no signal detected even after long exposure; ND, not determined. The relative intensity of the immunoblotting signals was the average of several experiments. ++ corresponds to the p27 amount found in extracts from normal human fibroblasts. Degradation activity: +++, completely degraded between 30 minutes and 3 hours incubation; ++, degraded between 6 hours and an overnight incubation; +, stable even after overnight incubation (see figure 5 for examples). +++ corresponds to the p27 degradation activity found in extracts from normal human fibroblasts (Pagano et al., *supra*).

Figure 7, is a table showing univariate analysis of potentially prognostic markers.

Figure 8, represents a table showing clinico-pathologic features of study population in relation to p27 status.

Figure 9, is a table showing multivariate analysis of potential prognostic markers by survival, using Cox models.

Detailed Description of the Invention

Progression through the cell-cycle is marked by a series of irreversible transitions that separate discrete tasks necessary for faithful cell duplication. These transitions are negatively regulated by signals that constrain the cell-cycle until specific conditions are fulfilled. Entry into mitosis, for example, is inhibited by incompletely replicated DNA or DNA damage. These restrictions on cell-cycle progression are essential for preserving the fidelity of the genetic information during cell division. The transition from G₁ to S phase, on the other hand, coordinates cell proliferation with environmental cues, after which the checks on the cell-cycle progression tend to be cell autonomous. Among the signals that restrict cell-cycle progression during G₁ are extracellular proteins which inhibit cell proliferation, growth factor or amino acid depletion, and cell-cell contact. Disruption of these signaling pathways uncouples cellular responses from environmental controls and may lead to unrestrained cell proliferation.

Eukaryotic cells, in general, require cyclin-dependent kinases (CDKs) for progression through G₁ and entry into S phase. In mammalian cells, both D-and E-type cyclins are rate limiting for the G₁ to S transition, and both reduce, but do not eliminate, the cell's requirement for mitogenic growth factors. Recently cyclins and CDKs have been found to be negatively regulated by either intracellular or extracellular signals that inhibit cell proliferation. In particular, various protein subunits have been identified and shown to inhibit the activity of CDKs. For this reason, these proteins have been named cyclin kinase inhibitors (CKIs).

The p27 protein (p27^{Kip1}) is a CKI protein implicated in the negative regulation of G₁ progression in response to a number of antiproliferative signals (Polyak et al. (1994) *Cell* 78:59. For example, studies in macrophages have linked cyclic AMP-induced growth arrest to an increase in the amount of p27 protein, whereas the antiproliferative drug rapamycin abrogates a small reduction in p27 abundance observed after colony-stimulating factor-1 stimulation (Kato et al. (1994) *Cell* 79:487). Likewise, interleukin-2-induced proliferation of T cells results in a decrease in the amount of p27 protein, an effect that can be prevented by addition of rapamycin (Bunce et al., (1994) *Leukemia* 8:595).

The p27 protein was first identified as an activity present in extracts derived from G1 cells able to inhibit CDK activities *in vivo*. The p27 gene was then cloned by the ability of its protein product to interact with CDKs and was found to share homology with p21 and block the cell cycle in G1 when overexpressed in mammalian cells. The primary regulation of p27 protein level during G1 is the result of ubiquitin-mediated degradation (Pagano et al. (1995) *Science* 269:682).

The CKIs have been suggested as potential antioncogenes since their function is often missing in transformed cells. For instance, p15 and p16 genes have been found mutated, deleted or inactivated by methylation in a large number of human malignancies. p21 is transcriptionally induced by the tumor suppressor p53, whose function is lacking in about 50%

of human tumors. It has been found that in p53 minus cells, p21 is poorly expressed and is not associated with CDKs. In contrast, the p27 gene analyzed by Southern blot and PCR-SSCP in a large number of human cancers and human cell lines showed no structural alterations of point mutations (Kawamat et al. (1995) *Cancer Res* 55:2266; Ponce-Castaneda et al. (1995) *Cancer Res* 55:1211; and Pietenpol et al. (1995) *Cancer Res* 55:1206).

On aspect of the instant invention is based on the discovery that the level of p27 protein can be correlated with progression of a hyperproliferative disorder. The level of p27 protein can be used predictively to evaluate whether a sample of cells contains cells which are, or are predisposed towards becoming, transformed cells. In general, the subject method can be characterized as including a step of detecting, in a sample of cells from the subject, the presence (level of) or absence of a p27 protein. As will be understood by those skilled in the art, the method of the present invention can be carried out using any of a large number of assay techniques for detecting destabilization of p27 protein, and importantly, provides the ability to discern between different molecular causes underlying aberrant cell growth, proliferation and/or differentiation.

Moreover, the subject method can be used to assess the phenotype of cells which are known to be transformed, the phenotyping results being useful in planning a particular therapeutic regimen. For instance, as the appended examples suggest, absence or low p27 protein expression is a powerful negative diagnostic and prognostic marker for a variety of cancerous diseases. The loss of p27 protein can be utilized in decisions regarding, e.g., the use of more aggressive therapies.

Prognosis in clinical cancer is an area of great concern and interest. It is important to know the aggressiveness of the malignant cells and the likelihood of tumor recurrence in order to plan the most effective therapy. Breast cancer, for example, is managed by several alternative strategies. In some cases local-regional and systemic radiation therapy is utilized while in other cases mastectomy and chemotherapy or mastectomy and radiation therapy are employed. As known in the art, treatment decisions for individual breast cancer patients can be based on, for example, the number of axillary lymph nodes involved with disease, estrogen receptor and progesterone receptor status, the size of the primary tumor, and stage of disease at diagnosis. It has also been reported that DNA aneuploidy and proliferative rate (percent S-phase) can help in predicting the course of disease (Dressler et al., (1988) *Cancer* 61:420; and Clark et al., (1989) *N. Engl. J. Med.* 320:627). The subject method provides a means for accurately predicting the course of disease for breast cancer patients. The ability to detect destabilization of the p27 protein can facilitate separation of patients with good prognosis, e.g., who will need little to no further therapy, from those more likely to recur who might benefit from more intensive treatments. This index can be combined with other prognostic methods.

This is particularly true in the case of breast cancer which has not progressed to the axillary lymph nodes ("node negative"). There is now evidence in prospective randomized clinical trials that adjuvant endocrine therapy and adjuvant chemotherapy beginning immediately after surgical removal of the primary breast tumor can be of benefit in some of these node-negative patients. This has led to recommendations in the art that most if not all node-negative breast cancer patients should be considered for some form of adjuvant therapy. But since the majority (approximately equal to 70%) of these patients enjoy long-term survival following surgery and/or radiotherapy without further treatment, it may be inappropriate to recommend adjuvant therapy for all of these patients. As described in the appended examples, the subject method can be used to distinguish those node-negative patients on the basis of significantly elevated or reduced risk of mortality, and suggests that this index may be useful in determining which patients would benefit from, e.g., continued and/or more aggressive therapy (such as adjuvant therapies).

It will also be appreciated that the subject method provides a procedure for predicting tumor recurrence in cancer patients in general, once the primary tumor is detected. The present invention is a significant step in the ability to predict with some confidence the likelihood of cancer recurrence. It is clear from the extensive studies on the p27 protein that it has an important physiological role, but until now no one has been able to relate its cellular levels or presence to clinical manifestations of dysfunction. Now the survival risk to cancer patients can be better assessed and aggressive therapies applied as indicated to those in high risk groups.

The term "prognosis" is art recognized and, as set out above, concerns the likelihood that an individual may suffer occurrence, relapse or distant relapse of cancerous disease. Relapse is the recurrence of tumor growth due to propagation of tumor cells remaining in the host after treatment, new tumor cell development, or the like. Distant relapse concerns tumor dissemination such that tumor growth occurs at a site distant from the site of the original tumor. Of additional interest in the case of disease relapse is the length of the relapse-free survival time. Relapse-free survival time is the period between either surgical removal of the tumor or the suppression or mitigation of tumor growth and the recurrence of cancerous disease. Prognosis may be affected by various criteria such as histological type, tumor grade, tumor size, ploidy, and expression of certain hormone receptors such as estrogen receptor, and, according to the present invention, the stability of p27. These criteria provide some guidance in determining the need for and efficacy of subjecting the patient to various cancer therapies, such as irradiation, adjuvant therapy or surgical procedures such as mastectomy in the case of breast cancer.

To further illustrate the subject method, the appended examples describe that levels of p27 protein, but not levels of p27 mRNA, are decreased in a variety of different tumor cells. For example, we have found that normal colonic mucosa, and well to moderately differentiated

(less aggressive) adenocarcinomas showed a strong p27 nuclear signal. In contrast, most of the poorly differentiated, highly aggressive adenocarcinomas showed a very low percentage of p27 positive cells. Furthermore, a highly significant correlation between the presence of p27 and survival of the patient was found. No correlation between the mitotic index of a particular tumor and p27 expression was found. Likewise, evaluation of biopsied tissue of a series of patients illustrates that the level of p27 protein provides a diagnostic and prognostic marker for breast cancers.

The appended examples describe that levels of p27 protein, but not levels of p27 mRNA, are decreased in a variety of different tumor cells. For example, we undertook an immunohistochemical analysis of p27 protein levels in breast ductal carcinomas, and found that low levels of p27 ($p=0.01$) were strongly predictive of increased mortality, both before and after adjustment for other clinical and pathological characteristics. Distant relapse-free survival, which is defined as survival without formation of tumors distant from the original site, is significantly inversely correlated with the combined analysis of the percent of p27 minus tumor cells. This enabled us to subdivide women with localized, node-negative disease into groups with either significantly elevated or reduced risk of mortality, and suggested that this index may be useful in determining which patients would benefit from more aggressive therapy.

The subject method is applicable to the diagnosis/prognosis of such breast cancer types as ductal, mucinous, lobular and the like. The cancer may be detected at any stage of tumor development, including hyperplasia, *in situ* and the like. The subject invention finds particular application for diagnosis where the patient carries an axillary lymph-node negative, ductal carcinoma of the breast.

As set out above, detection of p27 stability may serve as a marker for the presence of cancerous cells and also allow for determination of the prognosis of the patient carrying the tumor. The subject method can also be used to augment the detection and/or prognosis of such solid tumors as, for example, carcinomas (particularly epithelial-derived carcinomas) of such tissues as ovaries, lung, intestinal, pancreas, prostate, testis, liver, skin, stomach, renal, cervical, colorectal, and head and neck; melanomas; and sarcomas such as Kaposi's sarcoma and rhabdomyosarcoma. In preferred embodiments, the subject method is used to assess a malignant or pre-malignant epithelial carcinoma.

The diagnostic methods of the subject invention may also be employed as follow-up to treatment, e.g., quantitation of the level of p27 protein may be indicative of the effectiveness of current or previously employed cancer therapies as well as the effect of these therapies upon patient prognosis.

Accordingly, the present invention makes available diagnostic assays and reagents for detecting loss of p27 protein from a cell in order to aid in the diagnosis and phenotyping of

proliferative disorders arising from, for example, tumorigenic transformation of cells, or other hyperplastic or neoplastic transformation processes, as well as differentiative disorders, such as degeneration of tissue, e.g. neurodegeneration.

While not wishing to be bound by any particular theory, the inventors understand that the inactivation of the p27 protein occurs post-transcriptionally via an increase in its degradation due to, e.g., deregulation of the ubiquitin-proteasome pathway. As set forth in co-pending application USSN 08/486,663 (incorporated by reference herein), the p27 protein is an apparent substrate for ubiquitin-mediated proteolysis. According to this understanding, the rate of degradation of the p27 protein can be increased in particular cells, e.g., the protein is destabilized, leading to a net loss of p27 protein in the cytoplasm/nucleus of the cell.

By the present method, there is provided a method for evaluating an individual's risk (e.g., likelihood) of having or developing a disorder marked by aberrant proliferation or dedifferentiation. Where such disorders have already been diagnosed, the present method, by facilitating careful phenotyping of transformed cells, can improve the choice of intervention strategies by clinicians. For instance, the aggressiveness of the therapy to be used may be influenced by the knowledge of whether loss of regulation of proliferation or differentiation is caused by destabilization of a CKI protein, or other genetic abnormality.

Moreover, from the paradigm which develops from observing such destabilization to the p27 protein, another aspect of the present invention relates to diagnostic assays and reagents for detecting similar destabilization of other CKI proteins. Thus, another aspect of the present invention concerns an assay for detecting destabilization of such CIP proteins as p21^{CIP1} and p57^{KIP2}, as well as the *INK4* proteins such as p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19/p20^{INK4d}. For sake of clarity, the subject method and reagents will be described with reference to the p27 protein, those of skill in the art understanding that other CKI proteins can be similarly monitored, e.g., by use of antibodies, nucleic acid probes, etc. for detecting such CKI proteins and genes therefor.

I. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

An "inhibitor of CDK activation" refers to a naturally occurring protein which interacts with a cyclin dependent kinase and prevents activation of a kinase activity of the CDK either by, for example, inhibiting formation of CDK complexes including regulatory subunits, inhibiting interaction of the CDK subunit with activating kinases or phosphatases, inhibiting substrate binding, inhibiting ATP binding, and/or inhibiting conformational changes required

for enzymatic activity. Accordingly, such inhibition may be by a direct, competitive mechanism, or by an indirect, non- or uncompetitive mechanism.

To this end, the term "CKI protein" refers to a protein which is an inhibitor of CDK activation. Exemplary CKI proteins include members of the *INK4* family, such as p16^{INK4A} or p15^{INK4B}, and members of the *CIP/KIP* family, such as p21^{CIP1}, p27^{KIP1}, and p57^{KIP2}.

The term "*CIP/KIP* protein" refers to members of another CKI protein family which includes p27^{KIP1} (Polyak et al. (1994) *Cell* 78:67-74); p21^{CIP1} (WAF1/SDI1/CAP20; Xiong et al. (1993) *Nature* 366:701-704); and p57^{KIP2} (Lee et al. (1995) *Genes Dev.* 9:639-649; and Matsuoka et al. (1995) *Genes Dev.* 9:650-662). In addition to the functional characteristic of CDK inhibition, the *CIP/KIP* proteins each have a CDK inhibitory motif (a CDK-binding motif) of about 50 amino acids, referred to herein as a "p21/p27" inhibitory domain, which is conserved in members of the *CIP/KIP* family.

The term "*INK4* protein" refers to a family of structurally related CDK inhibitors characterized by a fourfold repeated ankyrin-like sequence (Elledge et al. (1994) *Curr. Opin. Cell Biol.* 6:874-878), and the ability to bind to CDKs, especially CDK4 and CDK6. Exemplary members of this protein family include p16 (*INK4A*/MTS1; Serrano et al (1993) *Nature* 366:704-707); p15 (*INK4B*; Hannon et al. (1994) *Nature* 371:257-261); p18 (*INK4c*; Guan et al. (1994) *Genes Dev.* 8:2939-2952) and p19/p20 (*INK4d*; Chan et al. (1995) *Mol. Cell Biol.* 15:2682-2688; and Hirai et al. (1995) *Mol. Cell Biol.* 15:2672-2681).

A "cyclin dependent kinase" or "CDK" are art recognized terms referring to protein of the family of proteins which include catalytic subunits of cyclin/CDK complexes. Exemplary CDK proteins include CDC2, CDK2, CDK3, CDK4, CDK5, CDK6 and CDK7. The sequence for wild-type CDK protein can be found, in GenBank.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" refers to a nucleic acid comprising an open reading frame, e.g., encoding a CKI protein, including (optionally) intron sequences. In preferred embodiments, the nucleic acid is DNA or RNA.

The terms protein, polypeptide, and peptide are used interchangeably herein.

The phrase "aberrant modification or mutation" of a gene refers to such genetic lesions as, for example, deletion, substitution or addition of nucleotides to a gene, as well as non-wild type splicing of mRNA transcribed from the gene. "Mis-expression" of a gene, on the other

hand, refers to aberrant levels of transcription of the gene relative to those levels in a normal cell under similar conditions.

The terms "reduced", "destabilized", "decreased" or the like, e.g., which denote quantitative differences between two states, refer to at least statistically significant differences between the two states. For example, "assessing whether the p27 protein is destabilized in the test cells" means that the level of p27 protein in the test cells is slower by at least a statistically significant amount relative to a normal cell. Such terms are also applied herein to, for example, rates of cell proliferation, levels of expression, and the like.

The term "p27-minus" refers to a cell phenotype wherein the cell possess a reduced cellular amount of the p27 protein relative to a normal cell of similar tissue origin. For example, a p27-minus cell may have less than 50%, 25%, 10%, or 5% of the p27 that a normal control cell,

The term "aberrant hyperproliferation" refers to proliferation of cells which is undesired, e.g., such as may arise it due to transformation and/or immortalization of the cells, e.g., neoplastic or hyperplastic, for purposes of wound healing, cosmetic, etc.

The term "aberrant dedifferentiation" refers to loss of differentiation of cells of a tissue such that the afflicted tissue losses at least a portion of the normal phenotype and function for animal at that development stage. For example, adult tissue undergoing aberrant dedifferentiation will be characterized by loss of at least a portion of the functional performance of that tissue in an adult organism.

The term "aberrant apoptosis" refers to unwanted cell death caused by apoptosis, e.g., as may occur in a variety of degenerative disorders, including such neurodegenerative disorders as Alzheimer's disease and Parkinson's disease.

The term "patient" refers to an animal, preferably a mammal, including humans as well as livestock and other veterinary subjects.

II. Diagnostic Assays

As set out above, one aspect of the present invention relates to diagnostic assays for determining, in the context of cells isolated from a patient, if the level of a p27 protein is significantly reduced in the sample cells. In particular, the assay evaluates the level of p27 protein in the test cells, and, preferably, compares the measured level with p27 protein levels detected in at least one control cell, e.g., a normal cell and/or a transformed cell. In general, the assay of the instant application detects destabilization of the p27 protein which decreases the level of protein in the cell, particularly in the nucleus.

Of particular importance to the subject invention is the ability to quantitate the level of p27 protein as determined by the number of cells associated with a normal or abnormal p27 level. The number of cells with a particular p27 phenotype may then be correlated with patient prognosis. In one embodiment of the invention, the p27 phenotype of the lesion is determined as a percentage of cells in a biopsy which are found to have abnormally low levels of p27 protein. Such expression may be detected by immunohistochemical assays, dot-blot assays, ELISA and the like. In general, it will be important that a significant number of cells in a section be examined, since tumors with only a small fraction of p27-minus cells could otherwise be scored p27 positive. For instance, a significant number of examined cells may comprise at least all the cells contained within 3 to 6 fields of a tissue sample observed at a magnification of 100x. As the number of cells examined in a tissue sample increases, the confidence one may have in classifying a sample as p27-minus increases.

To further illustrate, the presence of the p27 protein in a sample of cells can be determined by immunoassays or histochemical staining employing antibodies reactive with the p27 protein, e.g., antibodies specifically reactive with the p27 protein of SEQ ID. No. 2. Antibodies can be prepared conventionally (as described below) employing, e.g., the human p27 protein or antigenic fragments thereof, as the immunogen. For example, isolated p27 protein can be generated according to the methods described in PCT application PCT/US95/07361. Human p27 is not required, and antibodies raised against p27 from other vertebrates can be cross-reactive with the human protein.

Immunoassays are commonly used to quantitate the levels of proteins in cell samples, and many other immunoassay techniques are known in the art. The invention is not limited to a particular assay procedure, and therefore is intended to include both homogeneous and heterogeneous procedures. Exemplary immunoassays which can be conducted according to the invention include fluorescence polarization immunoassay (FPIA), fluorescence immunoassay (FIA), enzyme immunoassay (EIA), nephelometric inhibition immunoassay (NIA), enzyme linked immunosorbent assay (ELISA), and radioimmunoassay (RIA). An indicator moiety, or label group, can be attached to the anti-p27 antibody and is selected so as to meet the needs of various users of the method which are often dictated by the availability of assay equipment and compatible immunoassay procedures. General techniques to be used in performing the various immunoassays noted above are known to those of ordinary skill in the art.

Where tissue samples are employed, immunohistochemical staining may be used to determine the number of cells having the p27-minus phenotype. For such staining, a multiblock of tissue is taken from the biopsy or other tissue sample and subjected to proteolytic hydrolysis, employing such agents as protease K or pepsin. In certain embodiments, it may be desirable to isolate a nuclear fraction from the sample cells and detect the level of p27 in the nuclear fraction.

The tissue samples are fixed by treatment with a reagent such as formalin, glutaraldehyde, methanol, or the like. The samples are then incubated with an antibody, preferably a monoclonal antibody, with binding specificity for p27. This antibody may be conjugated to a label for subsequent detection of binding. Samples are incubated for a time sufficient for formation of complexes comprising p27 antigen and p27-specific antibody. Binding of the anti-p27 antibody is then detected by virtue of a label conjugated to this antibody. Where the anti-p27 antibody is unlabeled, a second labeled antibody may be employed, e.g., which is specific for the isotype of the anti-p27 antibody.

Where enzymes are employed as the detectable label (*infra*), the substrate for the enzyme may be added to the samples to provide a colored or fluorescent product. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art.

In one embodiment, the assay is performed as a dot blot assay. The dot blot assay finds particular application where tissue samples are employed as it allows determination of the average amount of p27 associated with a single cell by correlating the amount of p27 in a cell-free extract produced from a predetermined number of cells.

To achieve this, a single-cell suspension is produced from tissue sample taken from a patient, and the number of cells in each sample is determined. Tissues or cells which express p27, preferably tissues or cells which express a known amount of p27, may serve as a positive control and additionally may serve as a standard for comparison of results with test samples. Tissues or cells which lack p27 may be employed as a negative control.

It is well known in the art that single-cell suspensions may be produced from tissue in a variety of ways (e.g. incubation with EDTA in PBS, trypsin digestion, etc.). The number of cells in each sample may be determined by various conventional means, e.g. by Trypan blue or eosin staining. Cell-free extracts may be prepared from the single-cell suspensions by extraction with a detergent, such as Triton X-100, sonication or other conventional means known in the art. To provide for precise quantitative results, the protein concentration of each cell-free extracts may be determined by methods known in the art and dilutions from each extract employed in the assay.

Each sample may then be bound to a soluble support, such as nitrocellulose or the well of a microtiter plate. Following washing to remove unbound material, areas of the support which are not bound to extract components may be blocked by incubation with a blocking agent such as PBS containing bovine serum albumin (BSA) to reduce non-specific binding of subsequent reagents to the support. The samples may then be washed and subsequently incubated with an anti-p27 antibody, such as a monoclonal antibody, to allow for formation of complexes of p27 and the p27-specific antibody. Binding of the antibody may be detected and

analyzed as described above. The amount of p27 detected may be correlated with the number of cells in the original sample to determine the average amount of p27 protein per cell. The average amount of p27 protein per cell in the test sample may then be compared to the average amount of p27 protein per cell in standard samples and patient diagnosis/prognosis determined.

Still another exemplary embodiment of an immunoassay which can be adapted for detection of p27 protein levels is described in U.S. patent 4,499,183. Briefly, the '183 patent describes an immunoassay method in which the sample cells are contacted with a hypotonic solution carefully adjusted to swell the cells but not to result in substantial lysis prior to the completion of the assay, e.g., specifically the detection step. Swelling the cells results in an expanded cell size and larger cell membrane channels for facilitating antibody uptake. Thereafter, the cell membrane is rigidized and water is removed by standard fixation techniques. The cell is preferably first hydrated and then under appropriate conditions, reacted with a solution containing antibodies specific for the p27 protein. The antibody is advantageously labeled with a detectable label such as with a fluorochrome, metal particle, radioisotope or enzyme, or alternatively can be detected by an indirect immunofluorescence technique. The latter is accomplished by subsequent reaction of the cells previously reacted with the first antibody, with a second antibody specific for the first antibody. The second antibody is ideally labeled with a detectable label as above. Those antibodies not attached either directly or indirectly (i.e., by attachment through another antibody in turn attached to the antigen or antibody) to the intracellular p27 protein are removed by a washing process and the remaining cells analyzed for the presence of label. Detection of the label is in turn related to the presence of the intracellular p27 protein.

—It will be understood that other p27-binding molecules can be equivalently used in place of an antibody. For instance, CDK proteins can be used to detect/quantitate p27 protein.

In other embodiments, the level of p27 protein can be detected in cell lysates by chromatography or gel electrophoresis.

In yet another embodiment, the rate of degradation of p27 can be assessed in the sample cells. For instance, labeled p27 protein can be added to lysate from the sample cells and the rate of degradation monitored, e.g., by detecting fragments of the labeled protein or, alternatively, detecting ubiquitination of the ectopic p27. Likewise, proteolysis inhibitors can be added to the cells or cell lysates, and the rate of appearance of ubiquitination products of a heterologous or endogenous p27 protein can be detected. In each instance, the rate is compared to rates determined for control cells, e.g., cells of a known p27 phenotype. In other embodiments, the cells can be metabolically labeled and the rate of degradation of the endogenous p27 protein detected.

Used in conjunction with detection of p27 protein, the detection of p27 transcript in the cell sample can facilitate the determination of the molecular basis for loss of p27 protein in a

cell sample, e.g., to distinguish reduced transcription or translation from protein destabilization. Accordingly, the subject method may include a further step of determining the level of p27 mRNA in a sample of cells, e.g., by using a probe which hybridizes under stringent conditions to the p27 nucleic acid of SEQ ID No. 1.

The sample cells can be isolated to include a specific subset of phenotypes of cells from a given tissue, or can include be derived to include all or a substantial portion of cells representative of the tissue. Subsets of cells can be isolated, for example, by use of cell surface markers or careful sectioning of a tissue. For instance, in certain embodiments other antigens expressed by tumor cells may be detected, e.g., to determine the percentage of p27-minus cells in a given sub-population of cells from a sample. Tumor antigens of interest include breast carcinoma; prostate specific antigen (PSA); carcinoembryonic antigen (CEA); alpha-fetoprotein (AFP); CA125 antigen; soluble IL-2 receptor, progesterone receptor; estrogen receptor, and the like. The tumor-associated antigen CA-242 is expressed in the vast majority of colorectal tumors and is only weakly expressed or absent in normal colonic tissue. Other antibodies, against tumor cell markers associated with specific tumor types, which may be used in accordance with the method of the present invention include but are not limited to HEA125 MAb, HT29-15 MAb (colorectal carcinoma); BCD-B4 MAb or NCRC-11 MAb (breast carcinoma); anti-PSA antibody or PD41 MAb (prostate carcinoma); ALT-04 MAb (lung carcinoma); B72.3 MAb, HMD4 MAb or COC183B2 (ovarian carcinoma); HEA-125 MAb, MM46 MAb, or 9.2.27 MAb (melanomas); MGB 2 MAb, or ZCE 025 MAb (gastric carcinoma); BW494 MAb (pancreatic carcinoma); CEB9 MAb (uterine cervical carcinoma); HSAN 1.2 MAb (neuroblastoma); HISL-19 MAb (neuroendocrinomas); TRA-1-60 MAb (seminoma).

III. Antibodies, Proteins and Nucleic Acids

PCT application PCT/US95/07361 describes antibodies, proteins and nucleic acids for detecting p27 protein and mRNA levels. Exemplary antibodies and nucleic acid probes are also known in the art for other CKI proteins and can be readily adapted for use in the subject method. For example PCT applications PCT/US95/04636, PCT/US95/16553 and PCT/US96/01643 describes exemplary antibodies, proteins and nucleic acid probes which can be used as reagents in the subject method for embodiments directed to detection of *ink4* proteins. Likewise, PCT applications PCT/US93/09945 and PCT/US96/04563 describes antibodies, proteins and nucleic acid probes which could be used in the present method to detect destabilization of p21^{cip1} and p53^{kip2}, respectively.

To illustrate the generation of anti-p27 antibodies, it is noted using peptides based on the cDNA sequence of the p27 protein (e.g., SEQ ID No. 2), anti-p27 antisera or anti-p27 monoclonal antibodies can be made using standard methods. A mammal such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., an

antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. For instance, a peptidyl portion of the protein represented by SEQ ID No. 2 can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization with a p27 antigen, anti-p27 antisera can be obtained and, if desired, polyclonal anti-p27 antibodies isolated from the serum. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) *Nature*, 256: 495-497), as the human B cell hybridoma technique (Kozbar et al., (1983) *Immunology Today*, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the p27-protein of interest and the monoclonal antibodies isolated. Synthetic antibodies, e.g., generated by combinatorial mutagenesis and phage display, are equivalents of antibodies generated by immunization...

The labeled antibody may be a polyclonal or monoclonal antibody. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, for example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. The detectable marker may be, for example, selected, e.g., from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factor. Methods of labeling antibodies are well known in the art.

Antibody fragments which contain the idiotype of a p27 antigen can be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments generated by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing disulfide bridges of the F(ab')₂ fragments.

Similar strategies can be employed to generate the equivalent antibodies and antibody fragments for other CKI proteins.

One skilled in the art will appreciate that the invention also encompasses the use of antigen recognizing immunoglobulin fragments. Such immunoglobulin fragments include, for example, the Fab', F(ab')₂, Fv or Fab fragments, or other antigen recognizing immunoglobulin fragments. Such immunoglobulin fragments can be prepared, for example, by proteolytic

enzyme digestion, for example, by pepsin or papain digestion, reductive alkylation, or recombinant techniques. The materials and methods for preparing immunoglobulin fragments are well known to those skilled in the art.

In addition, the immunoglobulin may be a single chain antibody ("SCA"). These can consist of single chain Fv fragments ("scFv") in which the variable light ("V[L]") and variable heavy ("V[H]") domains are linked by a peptide bridge or by disulfide bonds. Also, the immunoglobulin may consist of single V[H] domains (dAbs) which possess antigen-binding activity. See, e.g., Winter and Milstein, (1991) *Nature* 349:295; and Glockshaber et al., (1990) *Biochemistry* 29:1362.

Synthetic antibodies, e.g., generated by combinatorial mutagenesis and phage display, are equivalents of antibodies generated by immunization.

As described above, the subject method can include a step of determining the level of mRNA transcripts for a CKI protein, e.g., preferably to discern between loss of protein stability and decreased translation and/or transcription as a cause for low CKI protein levels. Thus, a diagnostic kit of the present invention will include a nucleic acid probe/primer which can specifically detect the presence or absence of a CKI DNA or RNA sequence. In other embodiments, the cellular nucleic acid can be detected by proteins which selectively bind certain nucleic acid sequences, e.g., such as DNA binding domain from transcription factors.

The present invention therefore provides a probe/primer comprising a substantially purified oligonucleotide, wherein the oligonucleotide comprises a region of nucleotide sequence which hybridizes under stringent conditions to at least 10 consecutive nucleotides of sense or antisense sequence of SEQ ID No. 1, or naturally occurring mutants thereof. In preferred embodiments, the probe/primer further comprises a label group attached thereto and able to be detected, e.g. the label group is selected from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

Such oligonucleotide probes can be modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775).

The sample nucleic which is analyzed by the subject method can be isolated from any cell or collection of cells, though preferably is obtained from the cells from the same tissue sample used to score for p27 protein levels. There are a variety of methods known in the art for isolating RNA from a cellular source, any of which may be used to practice the present method. The Chomczynski method, e.g., isolation of total cellular RNA by the guanidine isothiocyanate (described in U.S. Patent No. 4,843,155) used in conjunction with, for example,

oligo-dT strepavidin beads, is an exemplary mRNA isolation protocol. The RNA, as desirable, can be converted to cDNA by reverse transcriptase, e.g., poly(dT)-primer first strand cDNA synthesis by reverse transcriptase, followed by second strand synthesis (DNA pol I).

An ideal reagent kit in accordance with the subject would therefore comprise an antibody specific for p27. In certain embodiments, that antibody can be labeled for detection. Alternatively, the kit can include a second labeled antibody which can detect the first antibody. The kit may also include various controls and washing buffers.

As described above, in certain embodiments, such as where intact cells are to be contacted with antibody, the kit can include a swelling solution.

In still other embodiments, another antibody for a cell marker is provided as a means for identifying the general population of cells in which a p27 minus phenotype is to be detected.

The kit may also include a nucleic acid probe for detecting the presence of a p27 transcript or cDNA thereof. The present invention specifically contemplates any and all variations of these reagents, as well as similar kits for use in the subject assay as pertaining to other CKI proteins.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1

Absence of p27 as a negative prognostic marker in stage II colorectal carcinomas

Progression through the cell cycle is positively regulated by association of cyclin dependent kinases (Cdks), with regulatory cyclin subunits. Negative regulation is achieved by cyclin dependent kinase inhibitors (CKIs), such as p27, which bind to Cdks. While p27 mRNA transcription is consistent throughout the cell cycle, protein levels are regulated by ubiquitin-mediated proteolysis.

Molecular prognostic evaluation may influence the treatment of patients with colorectal carcinomas, particularly with stage II cancers. Because p27 is a potential tumor suppressor gene, we analyzed p27 expression in colorectal carcinomas to determine its prognostic value.

The level of p27 expression was analyzed by immunohistochemistry and in situ hybridization in archival sections from one hundred and seventy one patients with colorectal

carcinomas, operated from 1965 to 1993, (median follow up was 36 months). We also determined p27 degradation activity in a subset of tumors.

The level of p27 protein was found to be an independent covariate by cox analysis, and its absence, (15.6% of patients) or low levels (58% of the patients) increased the risk of death from stage II disease 4.1 ($P = 0.0004$) and 2.5 ($P = 0.0007$) fold, respectively. Similar results were found when all stages were combined. In keeping with in vitro findings, carcinomas with low/absent p27 protein displayed enhanced ubiquitin-associated proteolytic activity specific for p27.

The instant results suggest that, (i) absence or low p27 protein expression is a powerful negative prognostic marker in colorectal carcinomas, in particular in stage II tumors, and thereby may help to select patients who will benefit from adjuvant therapy; (ii) enhanced degradation might be a mechanism to eliminate p27 in tumor cells.

Introduction

Progression through the eukaryotic cell division cycle is regulated by a number of cyclin dependent kinases (Cdks) consisting of a regulatory cyclin subunit and a catalytic serine/threonine kinase subunit (for a review, see ¹). Several distinct cyclin-Cdk complexes have been identified and found to be active and required at various stages of the cell cycle. The activity of Cdks is tightly regulated by both activating and inactivating phosphorylation events (reviewed by ²). Recently, a third class of subunits has been identified and shown to inhibit the activity of Cdks. For this reason, these proteins have been named cyclin kinase inhibitors (Ckis) (see ³ for a review). Most known Ckis promote cell cycle arrest in response to antimitogenic extracellular signals. Others Ckis might function as intrinsic checkpoints of the cell cycle ensuring that cells pass the G1 restriction point and replicate their genomes only under appropriate conditions.

In mammalian cells, two families of inhibitors have been cloned and characterized so far based on sequence homology and specificity of interaction with Cdks. The first family includes p21 (also called CPI 1, Pic1, Sdi1 and Waf1; ^{4, 5, 6, 7, 8}), p27 (also called d Kip1 and Pic2; ^{9,10}), and p57 (also called Kip2; ^{11,12}). The second family includes p16 (also called Ink4A, Mts1, Cdkn2 and Cdk4i; ^{13,14}), p15 (also called Ink4B, Mts2; ^{14, 15, 16, 17}), p18 (also called Ink4C and Ink6A; ^{15,18}), and p19/p20 (also called Ink4D and Ink6B; ^{18,19,20}).

Recently, Ckis have been suggested as the products of potential antioncogenes since their function is often missing in transformed cells. For instance, p15 and p16 genes have been found mutated, deleted or inactivated by methylation in a large number of human malignancies (reviewed by ²¹). P21 is transcriptionally induced by the tumor suppressor p53 ^{6, 22} whose function is lacking in about 50% of human tumors ²³. It has been found that in p53 minus cells,

p21 is poorly expressed and is not in association with Cdks²⁴. In contrast, the p27 gene analyzed by Southern blot and PCR-SSCP in a large number of human cancers and human cell lines showed no structural alterations or point mutations^{25, 26, 27}.

p27 was first identified as an activity present in extracts derived from G1 cells able to inhibit Cdk activities in vitro^{28, 29}. The p27 gene was then cloned by the ability of its protein product to interact with cdks and was found to share homology with p21 and block the cell cycle in G1 when overexpressed in mammalian cells^{9, 10}. Mammalian p27 protein accumulates in cells treated with cAMP³⁰, lovastatin³¹, TGFB^{29, 32}, or rapamycin³³ and this increase is thought to be involved in the G1 block induced by these drugs. Furthermore, p27 has been found to be expressed at high levels in quiescent cells, probably playing an important role in maintaining cells in G0^{33, 34, 35}. Similarly, cells undergoing differentiation have elevated level of p27^{36, 37}. Unlike p21, which is primarily regulated at the level of transcription, the downregulation of p27 protein level during G1 is the result of ubiquitin-mediated degradation³⁸.

Despite the lack of alterations in the p27 gene, and because its protein abundance is regulated in at the post-translational level, we decided to analyze p27 message and protein expression in human tumors, specifically in colorectal carcinomas.

Material and methods

Patients. Paraffin blocks were recovered from one hundred and seventy one patients who were operated at the Deaconess Hospital for adenocarcinoma of the colon or rectum between 1965 and 1993. These patients, randomly chosen with respect to date of operation, site of tumor and other factors, belong to a subset of a database containing information about clinic and pathologic variables and outcomes of the Joint Center for Radiation Therapy, Boston, MA³⁹. Outcome was verified and updated through the Tumor Registry and Medical Record Departments at the Deaconess Hospital as well as through contacts with municipal governments for death certificates when appropriate. Cancerous tissues from these patients had been characterized in term of clinical and pathological variables.

The effect of clinical variables, like age, sex and site of primary cancer, was not significantly associated with univariate life table analysis: (i) 33% of the patients were age 74 years or older; (ii) Gender was evenly distributed with females accounting for 47% of the patient population; (iii) The distribution of the primary site of cancer shows that the majority, 71% were located in the colon as compared to the rectum.

As expected, the effect of the pathological variables, like grade, TNM stage, and nodal status, was significant when assessed by univariate life table analysis: (i) well differentiated, 83% moderately differentiated, and 10% of tumors were poorly differentiated. Patients with

poorly differentiated tumors had a median survival rate of 95 months while patients possessing moderately and well differentiated tumors median survivals were 140 and 180 months, respectively (Wilcoxon $P < 0.0323$); (ii) The patient population consisted of 21% stage I, 47% stage II, 32% stage III and the median survival rates were 247 months, 149 months, 73 months respectively (Wilcoxon $p < 0.0001$). Due to the extremely small subset of stage IV carcinomas (and less than 1% stage IV), accurate conclusions could not be drawn concerning survival; (iii) Patients with negative nodes had a median survival rate of 308 months while patients 1-3 or more than 3 positive nodes had median survivals of 44 and 14 months, respectively (Wilcoxon $p < 0.0001$).

p27 Expression. Formalin-fixed, paraffin-embedded 5 μ m sections were mounted on charged glass slides, deparaffinized, and rehydrated through graded alcohols. Immunohistochemistry and in-situ hybridization were performed by an automated processor (Ventana Gene II, Ventana Medical Systems, Tucson, AZ). Immunohistochemistry slides were subjected to microwaving in 10mmole/L citrate buffer, pH 6.0 (BioGenex, San Ramon, CA.) in a 750 W oven inside a pressure cooker for a 30 minute period. Slides were allowed to cool at room temperature for 30 minutes. p27 mouse monoclonal antibody (mAb) (Transduction Laboratories, #K25020) was applied for 24 minutes at 37°C at a concentration of 1:200 in the automated stainer. Steps performed by the instrument include blocking with normal horse serum, application of a secondary antibody conjugated to the avidin-biotin peroxidase complex, and visualization with 3,3 diaminobenzidine as a substrate with standardized development times. Identical reaction times permitted accurate comparison of all samples. The slides were counterstained with methyl green and coverslipped. A cocktail comprised of antibodies with no known human recognition site⁴⁰ was used as a negative control.

An osteosarcoma cell line MG-63 (obtained from the American Type Culture Collection) was used as a positive control. Following 48 hours of serum starvation (which is necessary to increase levels of p27,³⁵ cells from two confluent flasks were harvested, fixed in neutral buffered formalin for 8 hours, and paraffin-embedded. 5 micron sections of the cell block were utilized in each run as a positive control. Pre-absorption of the antibody with the protein used to generate it abolished p27 staining (Fig. 2).

For in situ hybridization, 1 μ g of recombinant plasmid containing the human p27 full length of human p27 was linearized using BamHI and XbaI to generate antisense and sense transcripts respectively. Digoxigenin-labeled riboprobes were made with T7 and SP6 polymerase for 1 hour at 37°C in 1 X transcription buffer (Promega, Madison, WI), 10 mM dithiothreitol (DTT), 40 U of ribonuclease (RNase) inhibitor, adenosine, cytosine and guanosine triphosphates (1 mM each), and a mixture of cold uridine triphosphate (UTP) and digoxigenin-UTP (6.5 and 3.5 mM, respectively, for a total concentration of 1 mM) (Boehringer Mannheim, Indianapolis, IN). Slide sections were digested with proteinase K (50

µg/ml) in 1 M Tris-EDTA buffer (pH 8) for 8 minutes at 37°C for 15 minutes in 50% formamide and 2 X saline sodium citrate (SSC) followed by a neat denaturation step at 80°C for 2 minutes. Hybridization was performed at 42°C for 3 hours with the application of 10 pM digoxigenin-labeled riboprobe in 100 µl of hybridization buffer (50% deionized formamide, 2 X SSC, 50% dextran sulfate, 10% SDS, and denatured herring sperm DNA 10 mg/ml) per slide under liquid coverslip (Ventana Medical Systems, Tucson, AZ). Four washes of SSC at 42°C, the most stringent of which was 0.1 X followed hybridization. Anti-Digoxigenin antibody (1:500) was applied for 28 minutes at 37°C followed by detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) for 12 minutes.⁴¹ The slides were counterstained with methyl green and coverslipped. Sense probes were used as controls. Duration and temperature of all steps were standardized by the automated in situ machine.

p27 scoring. p27 staining, for both immunochemistry and in situ hybridization, was evaluated blindly (without knowledge of the clinic and pathologic parameters) or outcome and scored for degree of p27 expression as either 0 (less than 1% cells stained), 1 (1-50% cells stained), or 2 (greater than 50% cells stained).

Statistical Methods. Survival curves were constructed using the method of Kaplan-Meier.⁴² Survival was measured from the date of surgery to the date of last follow-up or death. Survival was censored if the patient was still alive or died from other causes. Univariate survival curves were compared using a Wilcoxon procedure⁴³ chi square analysis and log rank analysis. A Cox proportional hazards model⁴⁴ was used to assess the simultaneous contribution of the following baseline covariates: age, gender, site, grade, nodal status, T stage, TNM stage, and p27 expression. The distribution of p 27 was compared to the distribution of each baseline covariate using the-Jonckheere-Terpstra-Test.⁴⁵

Degradation assay. 1 g of each frozen human tissue sample was sectioned and quickly homogenized at 15,000 rpm with a Brinkman Polytron homogenizer (PT 3000) in 1 ml of ice cold double distilled water.⁴⁶ The sample was frozen and thawed 3 times. The lysate was spun down at 15,000 rpm in a Beckman JA-20.1 rotor for 45 minutes in 4°C. The supernatant was retrieved and frozen at -80°C. This method of preparation of total extract preserves ubiquitinating enzymes.^{38,47}

Purified histidine-tagged p27 (p27-his₆, bacterially expressed and purified as in ³⁸) was incubated at 37°C for different times in 30 µl of degradation mix containing 100 µg of protein human tissue homogenates, 40 mM Tris-HCl (pH 8.0), 4 mM MgCl₂, and 1 mM DTT, 2 mM ATP, 10 mM creatine phosphokinase, 10 mM creatine phosphate and 5 µM ubiquitin. Degradation of p27 was analyzed by immunoblotting with p27 mAb. While depletion of the proteasome, obtained by ultracentrifugation,^{38,48} inhibited the degradation of p27 (Fig. 5 E), the addition of the following protease inhibitors was ineffective in blocking p27 degradation: phenyl-methyl sulfonyl fluoride (PMSF), 0.1 mM; leupeptin, 1 µg/ml; soybean trypsin

inhibitor, 10 µg/ml; L-1 Chlor-3-(4-tosylamido)-4 Phenyl-2-butanone (TPCK); L-1 Chlor-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride (TLCK); 10 µg/ml; aprotinin 1 µg/ml.

Immunoblotting. Conditions for immunoblotting have been previously described.⁴⁹ Proteins were transferred from gel to a nitrocellulose membrane (Novex) by wet blotting as described in ⁵⁰. Filters were subjected to immunoblotting using a chemiluminescence (DuPont-NEN) detection system according to the manufacturer's instructions.

Results

Anti-p27 antibody detects non proliferating cells of normal colon mucosa.

Normal tissue away from the tumor (> 1 cm) or derived from patients with no tumors were stained with a well characterized mAb to p27.³⁸ We observed a nuclear staining localized to the top layer of terminally differentiated cells of the crypts, while proliferating cells in the bases of crypts were not detected (Fig. 1B). All intramucosal T lymphocytes (Fig. 1B) and most ganglion cells in the intestinal plexus showed positive p27 staining (data not shown). In contrast, an antibody to Ki67, a nuclear protein widely used as marker of proliferating cells,^{51,52,53,54} detected only cells located in the bases of the crypts (Fig. 1E). The staining with anti-p27 antibody was specific since it was blocked by preincubation of the antibody with recombinant purified p27 (Fig. 1C) but not with an irrelevant protein (not shown). To confirm that the staining observed in colonic epithelium was due to P27, a total cell lysate from the same human colon sample was run on a polyacrylamide gel, transferred to nitrocellulose, and probed with the same mAb to p27 used for immunohistochemistry. Only a Mr 27,000 protein, migrating slightly faster than recombinant purified p27-his₆, was revealed (Fig. 1F). Finally, p27 localization was also analyzed by in situ mRNA hybridization. Again, p27 mRNA expression was isolated at the upper half of the crypts (Fig. 1D).

Levels of p27 in human primary colorectal adenocarcinomas.

No homozygous deletions or mutations of the p27 gene have been found so far in cell lines or in human tumors.^{25,26,27} We examined levels of p27 protein in a characterized series of human primary colorectal tumors. The study population consisted of a retrospective series of tissues from 171 patients treated between 1965 and 1993. Cancerous tissues from these patients had been characterized in terms of clinical and pathological variables as described in detail in Materials and Methods. Distribution and association with outcome in the present series is consistent with those seen in other reported series.⁵⁵

Immunohistochemical staining revealed that p27 expression was present in 86% of tumors. 54% of tumors displayed 1-50% of p27 positive tumor cells, while 32% of tumors

showed more than 50% positive cells. P27 cellular localization within the same tumor was variably seen either in the nucleus, in the cytoplasm or both. No correlation between mitotic index of the particular tumor and p27 expression was found (data not shown). All (100%) (n=19) well differentiated cancers had p27 staining, 90% (n=131) of moderately differentiated tumors and 80% (n=21) of poorly differentiated cancers showed p27 staining. However, while the majority of p27 positive poorly differentiated cancers (93%) displayed staining in less than 50% of tumor cells or no staining, 51% of well/moderately differentiated cancers displayed p27 staining in more than 50% of cells ($p < 0.0092$ Jickheere-Terpstra Test). These findings suggest a progressive loss of p27 with increasing grade. Figure 2 shows examples of well and poorly differentiated colon carcinomas (Fig. 2A and 2B, respectively) as well as a tumor displaying both components (Fig. 2C) stained with anti p27 antibody.

p27 expression is prognostic in colorectal carcinoma patients.

A significant positive correlation ($p < 0.0031$) existed between p27 expression and outcome. Patients who had $>50\%$ p27 staining had a median survival in excess of 241 months, while patients who had $<50\%$ p27 expression or no p27 expression had median survivals of 140 months and 53 months, respectively (Fig. 3A). Patients subdivided according to presence or absence of p27 immunoreactivity of tumor cells had median survivals of 151 months to 53 months, respectively (Fig. 3B). Significantly, patients in stage II who had $>50\%$ p27 staining had a median survival in excess of 219 months, while patients who had $<50\%$ p27 expression or no p27 expression had median survivals of 140 months and 53 months, respectively (Fig. 3C). Patients according to a proportional hazard model, p27 is an independent prognostic variable ($p < 0.0031$) after controlling for age, sex, histologic grade, stage, and site. The absence of p27 increases the relative risk of dying from colorectal cancer by almost 4 fold (relative risk 3.81).

High p27 degradation activity in colon carcinomas is directly correlated to low levels of p27.

When we compared immunohistochemistry and in situ hybridization results obtained in a random subset of 49 patients with colorectal tumors, a direct correlation between protein and mRNA levels was not always found. In fact 12% of the tumors were positive for mRNA but not for protein expression. In addition, 47% of cases expressing high levels of mRNA had less than 50% of p27 positive cells by immunohistochemistry. Fig. 4 shows two typical examples: the tumor in panels A and B was positive for both p27 mRNA and protein expression; the second (panels C and D) expressed p27 mRNA but not protein.

Cell cycle regulation of p27 levels, both in normal and transformed human cells, occurs neither through a transcriptional nor a translational mechanism, but by degradation via the ubiquitin-proteasome pathway.³⁸ The discrepancy observed comparing p27 mRNA and protein, prompted us to test whether the p27 degradation pathway was enhanced in tumors with low p27 levels. We selected 15 tumors representative of high, moderate and low/absent p27 expression. We homogenized these tissues and made a total cellular extract that we tested for the abundance of p27 degradation activity. Fig. 5 shows a typical kinetics of p27 degradation obtained by using different samples of different conditions. Figure 6 shows that tumors with high levels of p27, as detected by western blot, presented low or very low p27 degradation activity (measured as described in the Material and Methods) compared to tumors with low/absent p27. These latter types of tumors, indeed, showed a high or a very high activity. This degradation activity was not due to a non-specific increase in the activity of the ubiquitin-proteasome pathway in some tumors. In fact, other cell cycle proteins found to be degraded by the ubiquitin-proteasome pathway, specifically, p21 and cyclin A (S.W.T. and M.P., unpublished results), were degraded in a similar fashion with all the extracts we tested (Figure 6).

Discussion of Results

The Cki p21 is a transcriptional target of p53.⁶ In tumor cells lacking functional p53, p21 levels are low and p21 is not found in association with cyclin-dependent kinases.²⁴ Immunocytochemical studies have demonstrated that there is a global decrease in p21 expression in human tumors compared to normal tissues.⁵⁶ In 75% of tumor cell lines and in a large number of human tumors, the *p16* gene is deleted or mutated (reviewed by ²¹.) Similarly, members of the p16 family, p15 and p18, can be the targets of genetic alterations. Thus, transformation often involves the inactivation of Cdk inhibitors.

No homozygous deletions or mutations of the *p27* gene have been found so far in cell lines or in human tumors.^{25,26,27} Since colorectal carcinoma is one of the most common causes of death from cancer in the United States,⁵⁷ we decided to verify the expression of p27 in a characterized series of 171 human colorectal carcinomas from patients treated between 1965 and 1993.

We found that 93% of poorly differentiated cancers displayed less than 50% of p27 - positive tumor cells or no staining. In contrast, 51% of well/moderately differentiated cancers displayed greater than 50% of p27 - positive tumor cells ($p < 0.0092$ Jockheere-Terpstra Test). Furthermore, lack of p27 expression and poor p27 expression are associated respectively with an increase of 4.1 and 2.5 - fold risk of dying from stage II colorectal carcinomas. Currently, the prognosis of colorectal carcinoma that has been resected curatively depends mainly on the analysis of standard clinical and pathological variables.⁵⁸ However, while predicting outcome

in patients with stage I and IV cancer is easy, the present staging method creates broad groups of patients whose survival may range at least forty percent within stage II and III. The same is true for predicting outcome based on the state of differentiation of the tumor. In fact, while patients with poorly differentiated, anaplastic tumors have a very poor survival rate, it is difficult to predict outcome in patients with well/moderately differentiated cancers. Improved prognostication may derive by evaluating the expression of molecular markers in tumor samples (see ⁵⁹ for a review). The results presented in our study demonstrate that assessment of p27 expression can be used as an independent prognostic marker for stage II colorectal carcinomas and might be a useful adjunct to histopathologic grade, particularly for well/moderately differentiated cancers. Furthermore, our study suggests that adjuvant therapy, which benefits patients with stage III cancer, may also be appropriate for patients with stage II disease whose colorectal tumors do not express p27. Allelic loss of chromosome 18q and increase in levels of sucrase-isomaltase (SI)⁵⁹ have also a prognostic value in patients with stage II colon cancer.⁵⁵ However, compared to the evaluation of the status of chromosome 18q, p27 assessment has the added advantage of being of easier execution, and compared to SI, p27 has better prognostic value (data not shown).

When p27 mRNA and protein levels were compared, no direct correlation was found. Indeed 12% of tumor samples expressed p27 message but failed to express the protein and 47% of tumors were strongly positive for p27 transcript but had low levels of protein. Since cellular levels of p27 are regulated mainly at the level of degradation,³⁸ it is tempting to speculate that one way for a cell to eliminate p27 and obtain a growth advantage is to enhance p27 ubiquitin-proteasome mediated proteolysis. To verify this hypothesis, we analyzed 15 tumor samples for the abundance of p27 specific degradation activity. A straight correlation between amount of p27, detected both by western blot, and amount of p27 degradation activity was found. This difference was not due to a non-specific enhanced degradation activity in some tumors compared to others. In fact, while p27 degradation activity correlates with p27 abundance, p21 and cyclin A degradation activity was independent of the relative amount of these proteins expressed in the same tumors. In conclusion, we suggest that in colorectal carcinomas p27 is inactivated neither at the genomic level (unlike p15 and p16) nor at the transcriptional level (unlike p21), but post-transcriptionally via an increase in its degradation due to a deregulation of the ubiquitin-proteasome pathway. So far, this is the second example of increased protein degradation involved in transformation. Indeed, in cells infected by human papillomavirus 16 and 18, levels of the tumor suppressor protein p53 are lower than in non-infected cells because of the viral E6 protein-mediated targeting of ubiquitinating enzymes to p53.^{60,61,62,63,64} We are currently conducting experiments aimed at understanding whether the alteration of the ubiquitin pathway observed in colorectal carcinomas is due to an enhancement of p27 ubiquitination by ubiquitinating enzymes, or to inactivation of deubiquitinating enzymes which might limit the degradation of p27 by rapidly disassembling ubiquitinated intermediates.

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Example 2

The cell cycle inhibitor p27 is an independent prognostic marker in small (T1a,b) invasive breast carcinomas

Breast carcinomas <1cm in size (T1a,b) are being detected more frequently as a result of screening. Marker/s are needed to identify the subset of patients who could benefit from adjuvant therapy, since traditional prognostic parameters are either lacking (tumor size) or rare (nodal metastases). A retrospective series of 202 patients with stage T1a,b invasive breast carcinomas who presented from 1969-1994 was evaluated. The clinicopathologic features (age, histologic grade, extensive *in situ* carcinoma, hormone receptor status, nodal metastasis)

as well as microvessel density, and the expression of c-erbB-2, p53, MIB-1/Ki-67, were assessed. In addition, expression of p27, a cell cycle inhibitor and cdc25B, a cell cycle activator associated with decreased disease-free survival in node-negative breast cancer patients and a potential oncogene, were evaluated.

Nineteen patients in our series (18% of patients who had axillary dissection) had loco-regional lymph node metastases. 42% of them died of disease (median survival of 122 months) while mortality was 11% on node-negative patients (median survival 168 months) ($p=0.0055$). Patients with low p27 expression (<50% of positive tumor cells; 50% of patients) had a median survival of 139 months (17% mortality) (vs. 174 months and 9% mortality in the group with high p27) were found to be independent prognostic parameters by multivariate analysis with a relative risk of dying of disease of 4.9 ($p=0.0001$) and 3.4 ($p=0.0306$), respectively.

In patients with T1a,b breast carcinomas, nodal metastases and low p27 expression are powerful predictors of poor survival. p27 assessment by immunohistochemistry is simple, cost-effective, reproducible and can be performed on all primary tumors. In addition, it yields prognostic information in node negative patients. Assessment of p27 could be useful to identify patients with small invasive breast carcinomas who might benefit from adjuvant therapy.

Recent trends in breast cancer detection have resulted in identification of progressively smaller invasive breast cancer, as well as increased numbers of in situ cancers. Several studies have shown that in small invasive tumors of <1cm the incidence of lymph node metastasis is very low, ranging from 3% to 13% (18% of patients who had axillary node dissection in this study)¹. At present, much controversies exist as regards the need for axillary lymph node dissection and/or adjuvant therapy following excision of small invasive primary tumor. In this study, we examined most pathologic parameters previously shown to be important either in the prognostication or in predicting response to therapy. In addition, we examined some of the most important biological markers known to be associated with mammary oncogenesis^{2, 3, 4, 5, 6, 7}.

Regulation of cell division cycle progression in mammalian cells depends on the dequencial activation of a series of cyclin-dependent kinases (Dcks, review in⁸). Cdk activation requires (i) association with an activating subunit (cyclin); (ii) de-phosphorylation by a family of dual-specificity phosphatases called Cdc25 (for a review see⁹; and (iii) dissociation from a Cdk inhibitor subunit (Dki, review in¹⁰).

The Cki p27 is a potential tumor suppressor. In fact, p27 deficient mice develop pituitary tumors with 100% penetrance and display increased body size^{11, 12, 13}. In addition, the Adenoviral E1A¹⁴ and the Human Papilloma viral E7 (P. Jansen-Duerr, pers. comm. oncoproteins inactivate p27 by dissociating it from cyclin-Cdk complexes. However, in

contrast to traditional anti-oncogenes like the Ck15 p15 and p16¹⁵, no homozygous deletions and only rare mutations on the p27 gene have been found so far in cell lines or in human tumors^{16, 17, 18}. Since p27 abundance is regulated at the post-transcriptional level^{19, 20}, we reasoned that, even in presence of a wild type gene, tumors might obtain a growth advantage from lack of p27 due to an increase in its degradation as we previously proposed in colorectal cancers²¹. Therefore, we analyzed the expression of p27 protein to assess the relationship between lack of p27 expression and aggressive behavior in small invasive breast cancers.

A cohort of 202 patients with invasive carcinomas which measured <1cm (T1a,b) were studied. All statistics were carried out with survival as an end-point. Patients were examined for differences in tumor size (T1a vs. T1b), histologic grade (modified Bloom-Richardson), extensive intraductal component (EIC)²², presence or absence of lymph node metastases in patients who had axillary node dissection (Figure 7), local recurrences (home-and contra-lateral), type of treatment (radiation therapy, chemotherapy, tamoxifen), and type of surgery. We also evaluated and included in the analysis the expression of the proto-oncogene c-erb-B2, the tumor suppressor gene p53, the proliferation marker MIB-1/Ki-67, estrogen and progesterone (ER and PR) receptors, microvessel density (MVD) as a measure of angiogenesis, and of cdc25B²³.

Of all clinico-pathologic parameters considered, only patients with positive nodes had a significantly lower survival (Figure 7). Specifically, of the 19 patients with positive nodes (18% of the patients who underwent axillary dissection), 8 (42%) died of disease (median survival of 112 months). In contrast, mortality was 11% in node-negative patients (median survival 168 months)(Log-Rank $p=0.0006$; Wilcoxon $p=0.0055$).

ER< PR, p53, MVD, Ki-67/MIB-1, cdc25B, or c-erb-B-2 were not significantly associated with survival by univariate analysis (Figure 7). In contrast, the level of p27 expression was associated significantly with survival by actuarial analysis with a median survival of 174 months in patients whose tumors displayed high p27 (>50%p27 positive cells), and 139 months in tumors that had low p27 expression (<50%p27 positive cells)(Log-Rank $p=0.0042$; Wilcoxon $p=0.0233$). High and low p27 expression in patients was equally distributed in the positive and negative node groups (Figure 8). Significance was maintained when node positive patients were excluded (log-Rank $p=0.0179$; Wilcoxon $p=0.0252$). Even in the small subgroup of patients who had axillary node dissection but were found to be node-negative, the group with low p27 expression had a mortality of 14% compared to 8% in the group with high p27 expression, although this was not quite significant (Log-Rank $p=0.0704$; Wilcoxon $p=0.0526$). p27 expression was not directly associated (i.e. was statistically independent or unrelated as a variable) with Ki-67/MIB-1 indicating that lack of p27 expression was not simply reflecting proliferation. Similar results were found in colorectal carcinomas as well²¹.

When a Cox proportional hazards model was constructed with all of the *clinicopathological* variables (Figure 9, Model 1), positive nodal status was a significant covariate ($p=0.0010$; relative risk 4.9); whereas age, size (T1a or b), tumor differentiation or extensive intraductal component (EIC) were not. When a Cox proportional hazards model was constructed with all of the *biological* variables (Figure 9, Model 2), low expression of p27 (<50% of tumor cells) and low expression of PR (<10%) were significant covariates ($p=0.0306$; relative risk 3.4 for p27 and $p=0.0364$; relative risk 3.3 for PR). In addition, ER expression (<10% of tumor cells) was also significant although with a rather wide confidence interval. Of note, neither hormone receptor status showed significant association with survival by univariate analysis. Finally, cdc25B, p53, MVD, ki-67/M1B1, and c-erb-B-2 were not independent parameters. Although the presence of nodal metastases were significantly associated with poor prognosis with a 4.9 fold relative risk of death, these patients represented only a small portion of the population. Importantly, node-negative patients with poor outcome can be identified on the basis of their p27 status, thus providing a powerful prognostic tool that can be utilized in all patients.

In summary, p27 is a novel prognostic marker the expression of which is associated with a better outcome in patients with T1a,b breast carcinomas who undergo potential curative surgery. Low p27 expression correlates with decreased survival, and may be utilized in T1a,b node-negative patients to predict prognosis. Our study suggests that adjuvant therapy, which benefits patients with advanced breast cancer, may also be appropriate for patients with T1a,b carcinomas expressing little p27.

METHODS

Population Study

328 consecutive patients with stage T1 (defined as tumors less than 1 cm in greatest diameter as determined by macroscopic measurement) invasive breast cancers diagnosed between 1969 and April 1994, were identified. Patients with distant metastasis at time of diagnosis, exclusive *in-situ* disease, synchronous bilateral breast carcinomas or a history or previous malignancies were excluded from this study. 202 patients (mean follow-up 72.3 months, median follow-up 65.9 months), with sufficient archival tissues available for analysis were included in this retrospective study. Age at diagnosis, biochemical steroid hormone receptor levels (available in 133 patients), modalities of therapeutic interventions, time to recurrence or metastasis, length of overall survival and cause of death were recorded. The mean age of the patients was 60.9 years (median 62.0); 42 patients (21%) were below 50 years of age. Follow-up data were obtained from patients charts and Tumor Registry records. Death from cancer was accepted when confirmed by autopsy or when there was convincing evidence of disease. Of the patients who were node negative or node indeterminate, 53 received

adjuvant therapy (51% of which was radiation therapy alone). Of the 19 node positive patients, 1 received chemotherapy 5%, 1 radiation therapy (5%), 3 hormonal therapy (16%), 7 radiation and chemotherapy (37%), 1 radiation and tamoxifen (5%) and 2 radiation, chemo and hormonal therapy (11%). Four patients (21%) received no adjuvant therapy. 23% of patients with ER positive tumors in our series were treated with tamoxifen. The median tumor size was 0.66 cm (range from 0.1 to 1.0 cm). Tumors <than 5mm in size (T1a) made up 31% of the series. Distribution of different histologic grades and presence or absence of extensive intraductal component in the patient population are outlined in Figure 7. The majority of carcinomas were ductal type (139 or 69%); while 45 (22%) were tubular carcinomas, 11 (5%) were lobular, 6 (3%) were colloid carcinomas, and 1 was a metaplastic carcinoma. Eleven patients (5%) developed local disease recurrence while 19 (9%) had radiologically or histopathologically documented distant metastases prior to death. Twenty six patients (13%) died of disease or disease-related complications. One hundred two patients underwent axillary node dissection and 19 of these had nodal metastases.

A representative block of formalin-fixed, paraffin-embedded carcinoma was selected for immunohistochemical (*c-erb* B-2, p53, factor 8, Mib1, ER and PR, p27) and *in-situ* hybridization (csc 25B) studies.

Immunohistochemistry

Immunohistochemistry was performed on an automated processor (Ventana ES, Ventana Medical Systems, Tucson, AZ). Formalin-fixed, paraffin embedded 5u sections mounted on charged glass slides were deparaffinized and rehydrated through graded alcohol before being placed in the automated immunostainer. Specificity of the p27 staining was assessed by : i) pre-absorption of the antibody with the protein used to generate it abolished p27 staining; ii) a cocktail comprised of antibodies with no known human recognition site used as a negative control; iii) positive staining of serum-starved MG-63 osteosarcoma cells (obtained from the American Type Culture Collection) in each immunoperoxidase run. Steps performed in the immunostainer include blocking with normal horse serum, application of the primary antibody, a biotinylated secondary antibody, and visualization with diaminobenzidine substrate, with standardized development time (allowing reproducible comparison between samples and between runs). The primary antibodies used were: monoclonal anti-*c-erb* B-2 (Ciba-Corning, Alameda, CA) dilution 1:150, incubation time 20 minutes; monoclonal anti-factor VIII related antigen (DAKP, Carpinteria, CA) dilution 1:100, incubation time 16 minutes, monoclonal anti-hormone receptors (ER and PK), (Ventura Medical Systems, Tucson, AZ); incubation time 32 minutes; monoclonal anti-p53 clone Ab-6 (Oncogene Science) dilution 1:500, incubation time 32 minutes; monoclonal anti-MIB-1, (Immunotech, Westbrook, ME) dilution 1:25, incubation time 16 minutes; monoclonal, anti-p27 (Transduction Laboratories, Lexington, KY) dilution 1:200, incubation time 24 minutes. A prior modified

antigen retrieval procedure was employed by microwaving deparaffinized slides in citrate buffer (pH 6.1) (Biogenex, San Ramon, CA) within a pressure cooker at power 10 (750W oven) for 20 minutes (ER and PR), power 5 for 16 minutes (p53 and MIB1, a power 7 for 16 minutes (p27).

In-situ hybridization

In-situ hybridization for cdc 25B was performed on the automated processor (Ventura Gen II, Ventana Medical Systems, Tucson, AZ) on 154 cases in which there was adequate preservation of mRNA. Digoxigenin-labeled probes were utilized for *in situ* hybridization as previously described²³.

Scoring of factors for statistical evaluation

Stained tumor sections were evaluated by estimating the percentage of invasive carcinoma cells positively stained by a primary antibody on the entire tissue section p27 staining, for both immunochemistry and *in situ* hybridization, was evaluated in a coded manner (without knowledge of the clinic and pathologic parameters or outcome) and scored independently for degree of p27 expression by two pathologist (M.L. and P.T.) At least 10 high power fields were counted. For *c-erb* B-2, a distinct brown membranous staining was scored as positive. For factor VIII, a distinct brown cytoplasmic staining in endothelial cells was considered positive. Areas with the highest vessel density were chosen at low magnification for counting. Microvessel density (MVD) was assessed by counting up to 3 fields at X200 field as previously described⁷. The range was 5 to 262 microvessels per X200 microscopic field, with a median value of 50. This value was used as a cutoff point. For all the other markers, namely p53, MIB-1, p27, ER and PR, a distinct brown nuclear staining was scored as positive. A cutoff value of <10% was used for p53, MIB-1, *c-erb*-B-2, and ER/PR, and <50% for p27, 50% of p27 positive tumor cells represented the median of this series. Sytosolic estrogen receptor assays were performed as previously described²⁴ cdc25B expression was assessed by *in situ* hybridization. Because expression of cdc25B, when present, was found throughout the tumor, sections were evaluated for intensity of staining which was scored as 0, 1, 3 to 3+. Only 3+ were considered overexpressors and this group was compared to 0, 1+, and 2+ combined.

Statistical analysis

The primary study outcome was survival, which was measured from the date of surgery to the date of last follow up or death. Survival was censored if the patient was still alive or died from other causes. Survival curves were constructed using the method of Kaplan-Meier²⁵. Univariate survival curves were compared using a Wilcoxon procedure²⁶ and differences between prognostic factors were tested for statistical significance with the log rank analysis²⁷ (Figure 9). A Cox proportional hazards model²⁸ for the risk ratio was used to assess the

simultaneous contribution of the following baseline covariates; age, grade, nodal status, presence or absence of EIC (Cox Model 1; Figure 9), and expression of the various markers (Cox Model 2; Figure 9) or all parameters combined (not shown). Ki-67/MIB-1 was excluded from Model 2 and the combined Cox because of 1) the scarcity of cases >10% positive cells (6/199) and 2) all deaths were clustered in the <10% group. All other covariates were retained in the model to illustrate lack of effect in the presence of other significant factors. The distribution of p27 was compared to the distribution of each baseline covariate using the Jonckheer-Terpstra Test²⁹ for doubly ordered data, Kruskai-Wallis³⁰ was used for single ordered data, and Fishers' exact test for categorical data. A p-value <0.05 was required for significance. Two-sided tests were performed throughout all analyses.

References for Example 2

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All of the above-cited references and publications are hereby incorporated by reference.

Sequence Listing

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 597 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..594

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG TCA AAC GTG CGA GTG TCT AAC GGG AGC CCT AGC CTG GAG CGG ATG	48
Met Ser Asn Val Arg Val Ser Asn Gly Ser Pro Ser Leu Glu Arg Met	
1 5 10 15	
GAC GCC AGG CAG GCG GAG CAC CCC AAG CCC TCG GCC TGC AGG AAC CTC	96
Asp Ala Arg Gln Ala Glu His Pro Lys Pro Ser Ala Cys Arg Asn Leu	
20 25 30	
TTC GGC CCG GTG GAC CAC GAA GAG TTA ACC CGG GAC TTG GAG AAG CAC	144
Phe Gly Pro Val Asp His Glu Glu Leu Thr Arg Asp Leu Glu Lys His	
35 40 45	
TGC AGA GAC ATG GAA GAG GCG AGC CAG CGC AAG TGG AAT TTC GAT TTT	192
Cys Arg Asp Met Glu Glu Ala Ser Gln Arg Lys Trp Asn Phe Asp Phe	
50 55 60	
CAG AAT CAC AAA CCC CTA GAG GGC AAG TAC GAG TGG CAA GAG GTG GAG	240
Gln Asn His Lys Pro Leu Glu Gly Lys Tyr Glu Trp Gln Glu Val Glu	
65 70 75 80	
AAG GGC AGC TTG CCC GAG TTC TAC TAC AGA CCC CCG CGG CCC CCC AAA	288
Lys Gly Ser Leu Pro Glu Phe Tyr Tyr Arg Pro Pro Arg Pro Pro Lys	
85 90 95	
GGT GCC TGC AAG GTG CCG GCG CAG GAG AGC CAG GAT GTC AGC GGG AGC	336
Gly Ala Cys Lys Val Pro Ala Gln Glu Ser Gln Asp Val Ser Gly Ser	
100 105 110	
CGC CCG GCG GCG CCT TTA ATT GGG GCT CCG GCT AAC TCT GAG GAC ACG	384
Arg Pro Ala Ala Pro Leu Ile Gly Ala Pro Ala Asn Ser Glu Asp Thr	
115 120 125	
CAT TTG GTG GAC CCA AAG ACT GAT CCG TCG GAC AGC CAG ACG GGG TTA	432
His Leu Val Asp Pro Lys Thr Asp Pro Ser Asp Ser Gln Thr Gly Leu	
130 135 140	
GCG GAG CAA TGC GCA GGA ATA AGG AAG CGA CCT GCA ACC GAC GAT TCT	480
Ala Glu Gln Cys Ala Gly Ile Arg Lys Arg Pro Ala Thr Asp Asp Ser	
145 150 155 160	
TCT ACT CAA AAC AAA AGA GCC AAC AGA ACA GAA GAA AAT GTT TCA GAC	528
Ser Thr Gln Asn Lys Arg Ala Asn Arg Thr Glu Glu Asn Val Ser Asp	
165 170 175	
GGT TCC CCA AAT GCC GGT TCT GTG GAG CAG ACG CCC AAG AAG CCT GGC	576
Gly Ser Pro Asn Ala Gly Ser Val Glu Gln Thr Pro Lys Lys Pro Gly	

180

185

190

CTC AGA AGA CGT CAA ACG TAA
 Leu Arg Arg Arg Gln Thr
 195

597

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 198 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Asn Val Arg Val Ser Asn Gly Ser Pro Ser Leu Glu Arg Met
 1 5 10 15
 Asp Ala Arg Gln Ala Glu His Pro Lys Pro Ser Ala Cys Arg Asn Leu
 20 25 30
 Phe Gly Pro Val Asp His Glu Glu Leu Thr Arg Asp Leu Glu Lys His
 35 40 45
 Cys Arg Asp Met Glu Glu Ala Ser Gln Arg Lys Trp Asn Phe Asp Phe
 50 55 60
 Gln Asn His Lys Pro Leu Glu Gly Lys Tyr Glu Trp Gln Glu Val Glu
 65 70 75 80
 Lys Gly Ser Leu Pro Glu Phe Tyr Tyr Arg Pro Pro Arg Pro Pro Lys
 85 90 95
 Gly Ala Cys Lys Val Pro Ala Gln Glu Ser Gln Asp Val Ser Gly Ser
 100 105 110
 Arg Pro Ala Ala Pro Leu Ile Gly Ala Pro Ala Asn Ser Glu Asp Thr
 115 120 125
 His Leu Val Asp Pro Lys Thr Asp Pro Ser Asp Ser Gln Thr Gly Leu
 130 135 140
 Ala Glu Gln Cys Ala Gly Ile Arg Lys Arg Pro Ala Thr Asp Asp Ser
 145 150 155 160
 Ser Thr Gln Asn Lys Arg Ala Asn Arg Thr Glu Glu Asn Val Ser Asp
 165 170 175
 Gly Ser Pro Asn Ala Gly Ser Val Glu Gln Thr Pro Lys Lys Pro Gly
 180 185 190
 Leu Arg Arg Arg Gln Thr
 195

We Claim:

1. A method for diagnosing a hyperproliferative disorder in a patient which disorder is associated with the destabilization of a CKI protein in cells of the patient, comprising: (i) ascertaining the level of CKI protein in a sample of cells from the patient; and (ii) diagnosing the presence or absence of a hyperproliferative disorder utilizing, at least in part, the ascertained level of CKI protein, wherein a reduced level of a CKI protein in the sample, relative to a normal control sample of cells, correlates with the presence of a hyperproliferative disorder.
2. A prognostic method for evaluating the aggressiveness and/or rate of recurrence of a disorder marked by aberrant hyperproliferation, aberrant dedifferentiation and/or aberrant apoptosis of cells, comprising (i) ascertaining the level of CKI protein in a sample of cells from a patient; and (ii) ascertaining the aggressiveness and/or risk for recurrence of the disorder, at least in part, from the ascertained level of CKI protein, wherein a reduced level of CKI protein in the sample, relative to a normal control sample of cells, correlates with a more aggressive form of the disorder and an increased risk of recurrence of the disorder.
3. A prognostic method for evaluating a cancer patient's risk of death due to cancer and/or recurrence of a cancer, comprising (i) ascertaining the level of CKI protein in a sample of cancer cells from the patient; and (ii) predicting the patient's risk of death and/or recurrence of a cancer utilizing, at least in part, the ascertained level of CKI protein, wherein a reduced level of CKI protein in the sample, relative to a normal control sample of cells, correlates with an increased risk of death and/or recurrence of a cancer.
4. The method of any of claims 1-3 wherein the CKI protein is a CIP/KIP protein.
5. The method of claim 4, wherein the CIP/KIP protein is p27^{kip1}.
6. The method of any of claims 1-3, wherein the patient is a human.
7. The method of any of claims 1-3, wherein the level of CKI protein is determined by immunoassay.
8. The method of any of claims 1-3, wherein the level of CKI protein is determined by chromatography or electrophoresis.
9. The method of claim 3, wherein the cancer is a cancer of an epithelial tissue.
10. The method of claim 3, wherein the cancer is a cancer selected from the group consisting of carcinomas, melanomas and sarcomas.

11. The method of claim 10, wherein the cancer is a carcinoma of a tissue selected from the group consisting of breast, ovaries, lung, intestinal, pancreas, prostate, testis, liver, skin, stomach, renal, cervical and colorectal.
12. The method of claim 10, wherein the cancer is a breast carcinoma.
13. The method of claim 12, wherein the breast carcinoma is a ductal carcinoma, a mucinous carcinoma or a lobular carcinoma.
14. The method of claim 3, wherein the cancer is a node-negative breast carcinoma.
15. The method of claim 1-3, comprising the further steps of ascertaining, in the sample of cells, the level of mRNA transcript which encodes the CKI protein, the level of transcript providing for determination of whether a reduced level of CKI protein in the sample cells, relative to a normal control sample of cells, is due to destabilization of the CKI protein or loss of expression of the CKI gene.
16. The method of claim 3, comprising the further step of applying a treatment to the patient as determined, at least in part, on the basis of the patient's risk of death and/or recurrence of the cancer.
17. A test kit for detecting destabilization of p27 protein comprising an antibody, or fragment thereof, which selectively binds to a p27 protein, and a nucleic acid probe which selectively detects p27 transcripts or cDNA thereof.
18. The kit of claim 17, wherein the antibody selectively binds to the p27 protein of SEQ ID No. 2.
19. The kit of claim 17, wherein the probe selectively hybridizes under stringent conditions to the nucleic acid of SEQ ID No. 1.
19. The method of claim 2 and the kit of claim 17, wherein at least one of the antibody and probe is labeled with a detectable label.
20. The method and kit of claim 19, wherein the label is selected from the group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.
21. A test kit for detecting p27 protein comprising an anti-p27 antibody, or fragment thereof, which selectively binds to a p27 protein, and a permeabilizing agent for permeabilizing a cell and permitting intracellular localization of the anti-p27 antibody, which permeabilizing agent does not cause significant lysis of the cell over a time period sufficient for detecting complexes formed between the anti-p27 antibody and p27 protein present in the cells.
22. The kit of claim 17 or 21, which kit further includes an antibody which selectively binds to and detects a second antigen expressed by cancer cells.

23. The kit of claim 17 or 21, which kit further includes an antibody which selectively binds to and detects a cell surface antigen expressed by only a portion of the cells of a patient.

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Figure 1



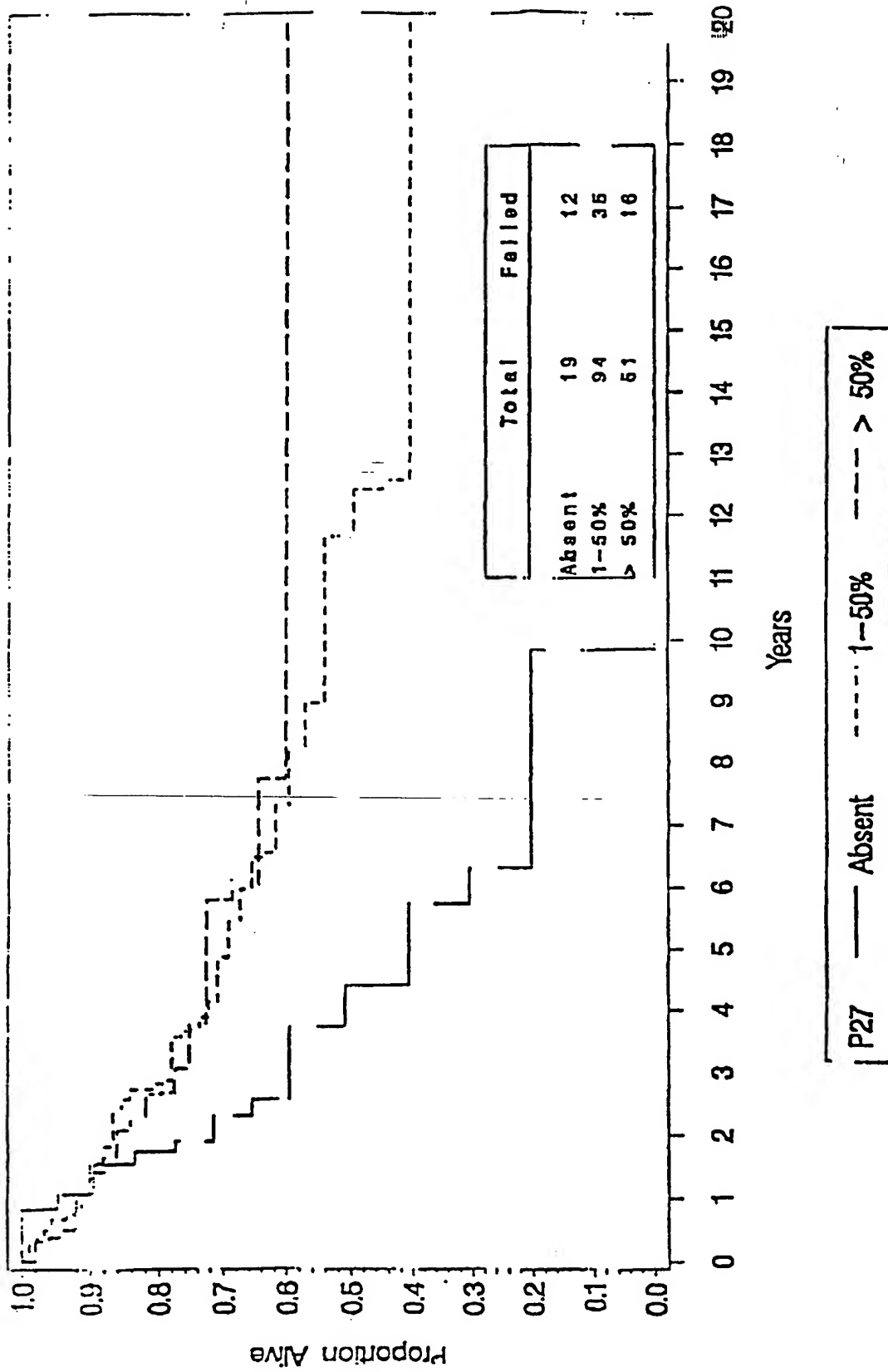
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Figure 2



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Figure 3A
NEDH Colorectal Cancer Survival (All P27 Data)
P27 Comparison for All Stages

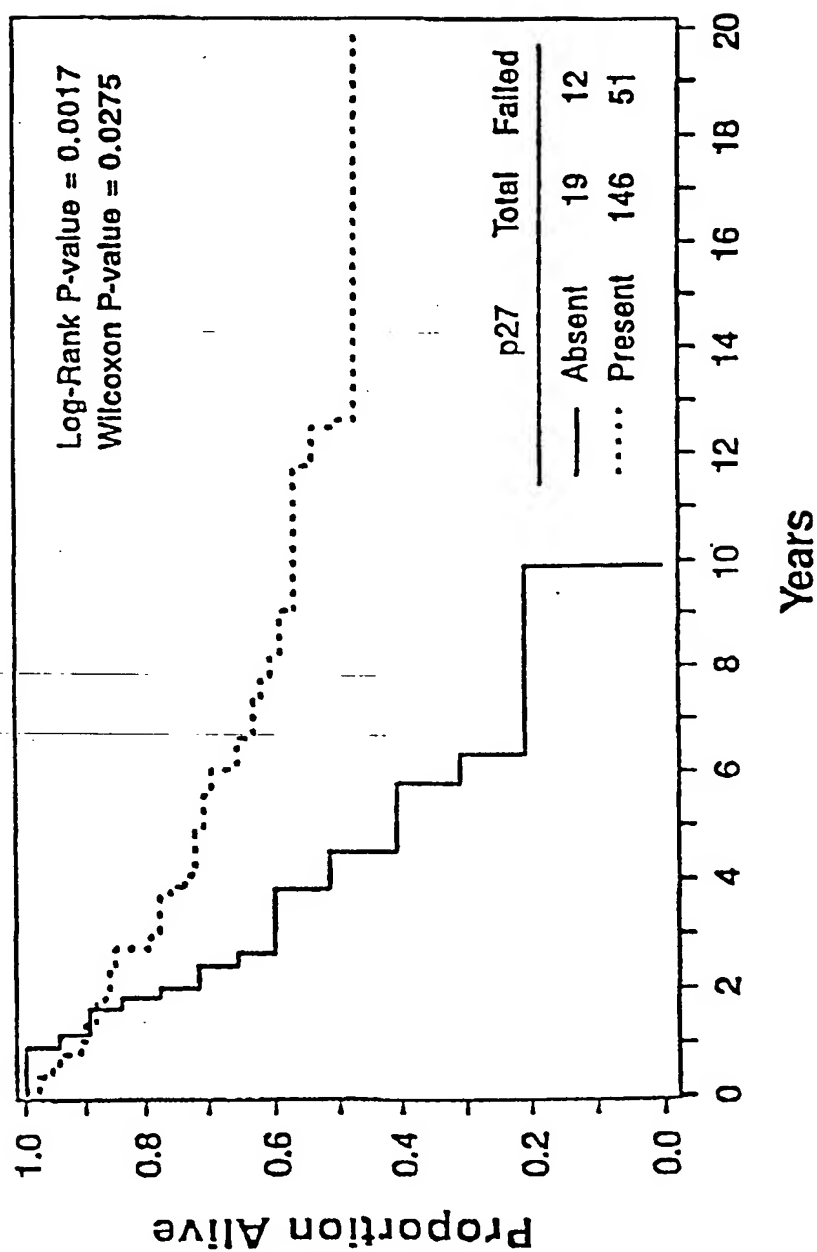


Log-Rank P-value = 0.0069, Wilcoxon P-value = 0.0927
 Medians (months): 53 vs 140 vs > 241

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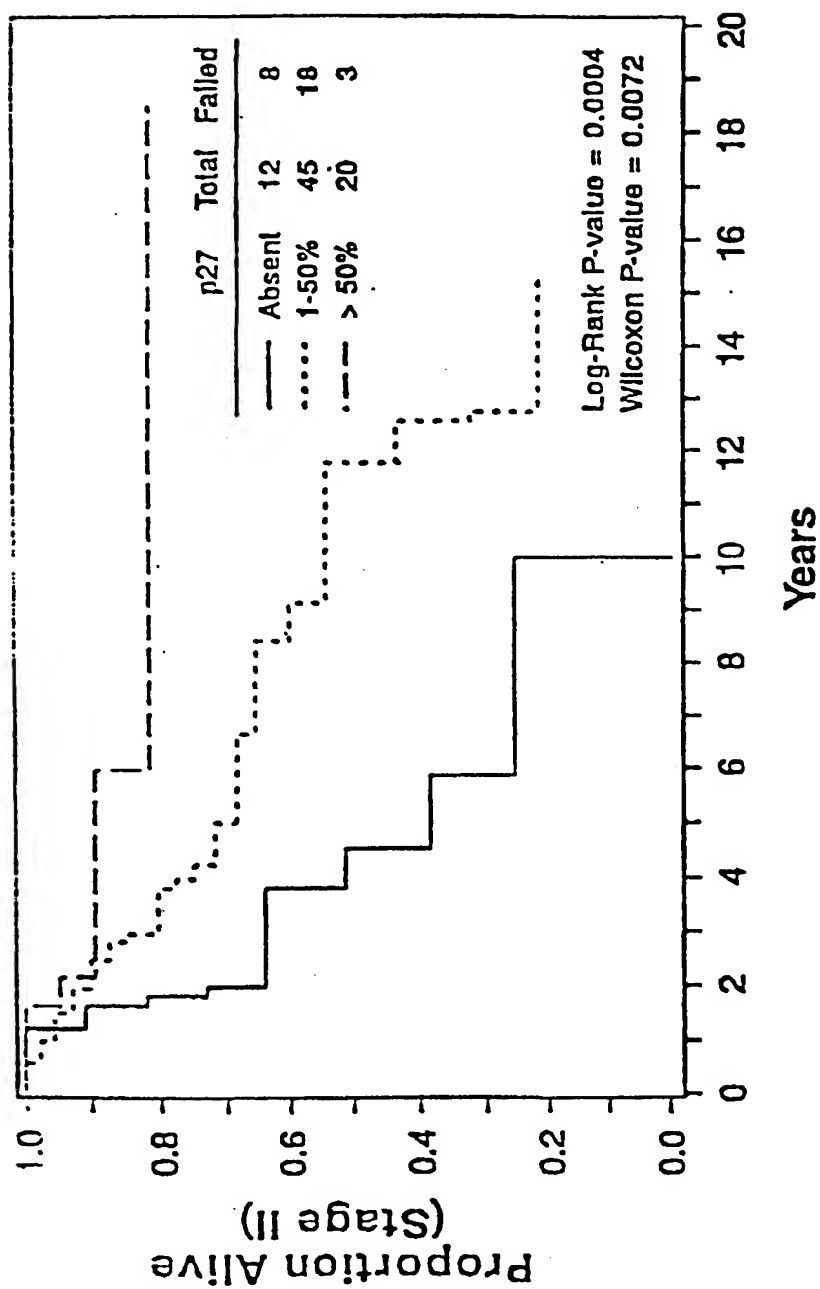
Figure 3B

NEDH Colorectal Cancer Survival (All p27 Data) p27 Comparison



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Figure 3C



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Figure 4

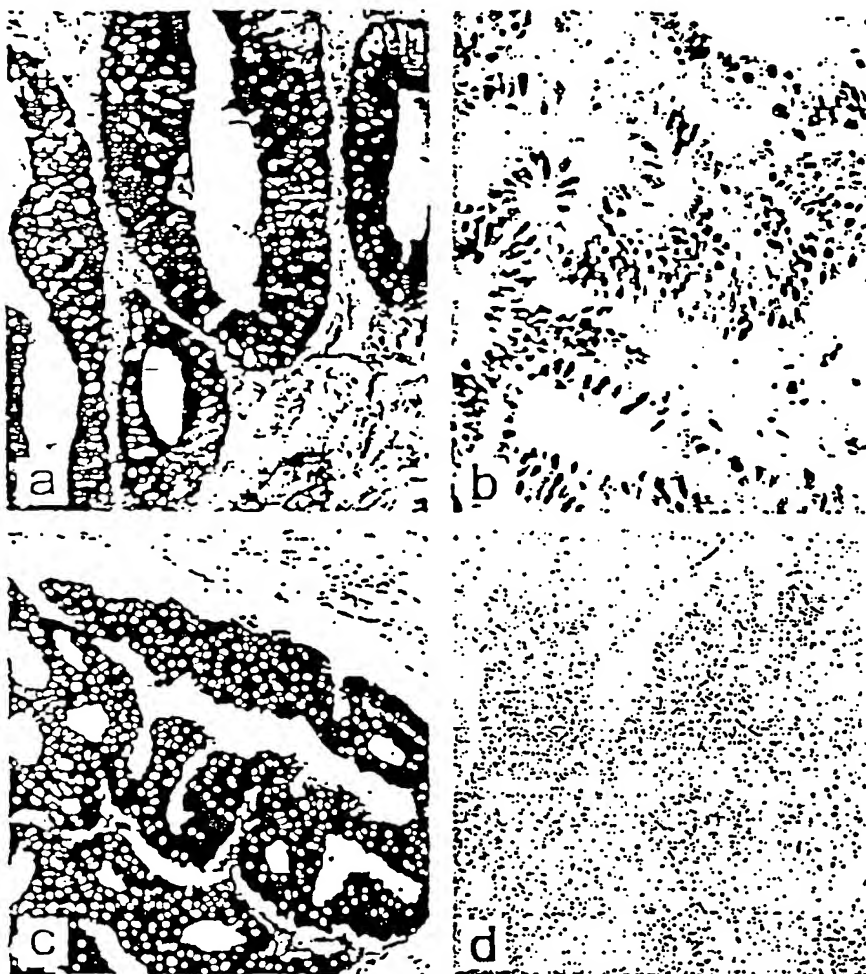
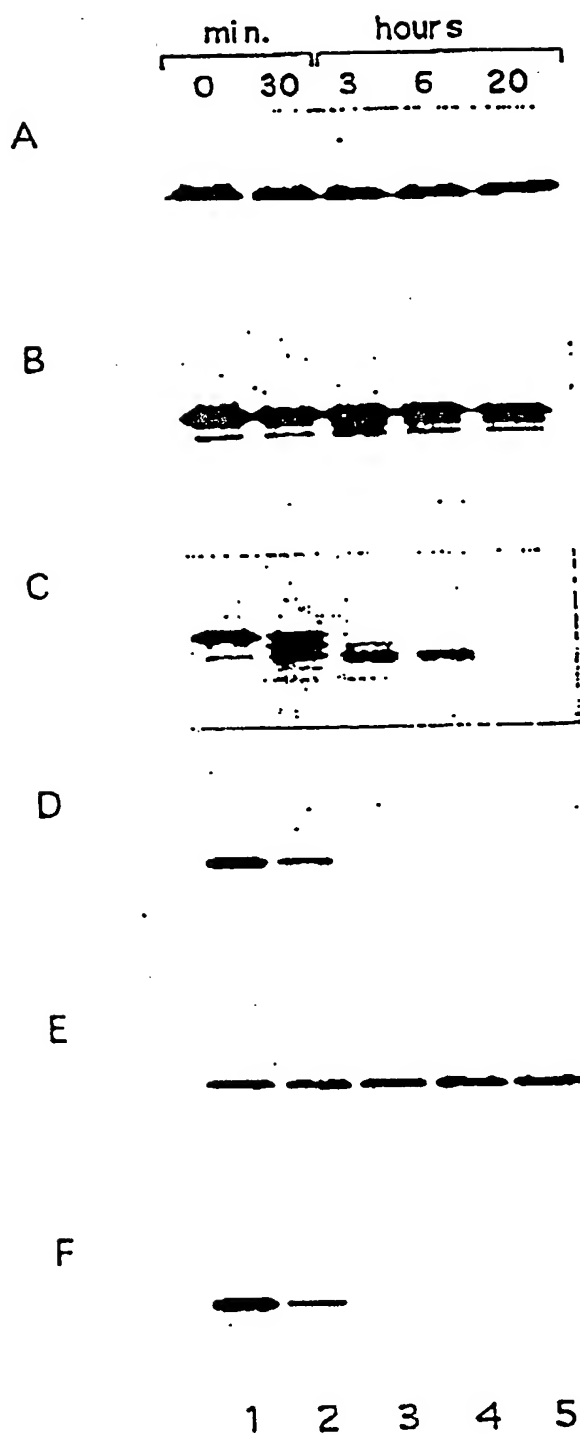


Figure 5

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Bank#	p27 levels	p27 degradation activity	p21 levels	p21 degradation activity	Cyclin A levels	Cyclin A degradation activity
90-258	-	++++	-	ND	+	ND
92-28	+	++++	+	++++	++	+++
91-69	+	+++	-	++++	+	++
92-49	+	+++	-	ND	+	ND
91-199	+	+++	-	++++	+	++
91-16	+	+++	ND	ND	ND	ND
91-13	++	++	+	++++	++	++
90-54	++	++	-	ND	+	ND
92-158	+++	++	-	++++	+	ND
92-10	+++	++	-	ND	+	++
92-11	+++	++	-	ND	+	ND
91-82	+++	+	+++	+	+++	++
91-93	+++	+	+	++++	++	++
95-8	+++	+	+	ND	+	ND
90-253	+++	+	++	++++	+++	++

Figure 6

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Figure 7

Markers (Total patients)	# of pt	Death from Disease		Cohort Survival (Median # of mths)	p-value
		N	%		
Tumor size (n=202)					
T1a	62	8	(13%)	174	0.9802 †
T1b	140	18	(13%)	168	0.7018 ¥
Histologic grade (n=202)					
I	70	8	(11%)	162	0.9791 †
II	89	13	(15%)	160	0.7550 ¥
III	43	5	(12%)	174	
EIC component (n=202)					
Pos (≥ 25%)	141	15	(11%)	168	0.2755 †
Neg (< 25%)	61	11	(18%)	174	0.4226 ¥
Lymph node metastases (n=102)†					
Yes	19	8	(42%)	112	0.0006 †
No	83	9	(11%)	168	0.0055 ¥
p27 immunostaining (n=202)					
< 50%	100	17	(17%)	139	0.0042 †
≥ 50%	102	9	(9%)	174	0.0233 ¥
cd25B score (n=154)					
< 3	122	13	(11%)	168	0.1803 †
= 3	32	5	(16%)	132	0.1348 ¥
c-erbB-2 immunostaining (n=200)					
< 10%	177	23	(13%)	162	0.1015 †
≥ 10%	23	2	(9%)	174	0.2463 ¥
p53 immunostaining (n=200)					
< 10%	179	21	(12%)	174	0.1941 †
≥ 10%	21	5	(24%)	168	0.4468 ¥
MB1 immunostaining (n=199)					
< 10%	193	26	(13%)	146	0.2948 †
≥ 10%	6	0	(0%)	168	0.4415 ¥
ER immunostaining (n=196)					
< 10%	56	6	(6%)	174	0.1650 †
≥ 10%	140	21	(13%)	168	0.4104 ¥
ER by cytosolic assay (n=133)					
< 10 fmol	78	10	(13%)	162	0.0806 †
≥ 10 fmol	55	4	(7%)	168	0.0390 ¥
PR immunostaining (n=194)					
< 10%	94	16	(17%)	174	0.0961 †
≥ 10%	100	10	(10%)	168	0.1735 ¥
Microvessel density (n=200)					
Low (< 50)	124	13	(10%)	168	0.6409 †
High (≥ 50)	76	12	(16%)	174	0.3963 ¥

p = †: Log-rank, ¥: Wilcoxon values † only patients who had axillary node dissection

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Figure 8

Markers	p27 Groupings		Total N
	N <50% %	N ≥50% %	
Number of Patients	100	102	202
Age (years)			
< 50	18 (18%)	24 (24%)	42
≥ 50	82 (82%)	78 (76%)	160
Tumor size (cm)			
T1a (≤0.5)	32 (32%)	30 (29%)	62
T1b (>0.5; ≤ 1.0)	68 (68%)	72 (71%)	140
Histologic grade			
I	37 (37%)	33 (32%)	70
II	43 (43%)	46 (45%)	89
III	20 (20%)	23 (23%)	43
HIC (% of tumor size)			
Pos (≥25%)	68 (69%)	73 (72%)	141
Neg (<25%)	32 (31%)	29 (28%)	61
Local recurrence			
Yes	1 (1%)	10 (10%)	11
(% contralateral)	0 (0%)	1 (10%)	
No	99 (99%)	92 (90%)	191
(% contralateral)	5 (5%)	6 (7%)	
Lymph node metastases			
Yes	12 (12%)	7 (7%)	19
No	88 (88%)	95 (93%)	183
Lymph node metastases with axillary dissection			
Yes	12 (26%)	7 (13%)	19
No	35 (74%)	48 (87%)	83

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Figure 9

Model 1:

Clinicopathologic Parameters	Category	Risk ratio	95 % confidence interval	p value #
Age (years)	0 = < 50, 1 = ≥ 50	1.508	0.598 - 3.802	0.3833
Positive lymph nodes	0 = No, 1 = Yes	4.877	1.900 - 12.523	0.0010
Tumor size	0 = T1a, 1 = T1b	1.034	0.4140 - 2.584	0.9421
Histologic grade I	0 = III, 1 = I	1.125	0.334 - 3.784	0.8495
Histologic grade II	0 = III, 1 = II	1.166	0.378 - 3.584	0.7901
EIC	0 = < 25%, 1 = ≥ 25%	1.325	0.557 - 3.155	0.5241

Model 2:

Biologic Parameters	Category	Risk ratio	95% confidence interval	p value #
c-erbB-2	0 = < 10%, 1 = ≥ 10%	1.684	0.1846 - 15.385	0.6444
p53	0 = < 10%, 1 = ≥ 10%	2.344	0.603 - 9.113	0.2190
p27	1 = ≥ 50%, 0 = < 50%	3.401	1.121 - 10.309	0.0306
cdc25B	0 = < 3, 1 = ≥ 3	1.740	0.532 - 5.687	0.3594
HR immunostaining	0 = < 10%, 1 = ≥ 10%	4.158	1.000 - 17.289	0.0500
FR immunostaining	1 = < 10%, 0 = ≥ 10%	3.289	1.079 - 10.101	0.0364
Microvessel density	0 = < 50, 1 = ≥ 50	2.260	0.813 - 6.281	0.1178

p value is calculated by Chi square